PLANT PHYSIOLOGY

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STUDIES IN THE METABOLISM OF CRASSULACEAN PLANTS: CHANGES IN THE COMPOSITION OF *BRYOPHYLLUM CALYCINUM* DURING GROWTH

GEORGE W. PUCHER, CHARLES S. LEAVENWORTH, WANDA D. GINTER, AND HUBERT BRADFORD VICKERY

(WITH TWENTY-ONE FIGURES)

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Certain peculiarities in the chemical composition of succulent plants, especially those belonging to the family Crassulaceae, have long attracted the attention of plant physiologists and chemists. As early as 1815, HEYNE (**4**) noted that the leaves of *Bryophyllum calycinum* (then known as *Cotyledon calycina*) were intensely sour to the taste in the morning but lost their sour taste toward evening, becoming bitter instead. The outstanding chemical studies of this phenomenon were made 60 years and more ago by ADOLPH MAYER (**7**) who associated the changes in taste with reciprocal diurnal alterations in the concentrations, respectively, of titratable acid and of sugar. He also demonstrated that oxygen is liberated during the deacidification process in light, even when carbon dioxide is excluded, and recognized what he thought to be an isomer of malic acid.

An extensive literature on what is commonly referred to as the crassulacean type of metabolism has since appeared. The papers before 1933 have been carefully reviewed by BENNET-CLARK (**1**) [see also EVANS (**3**), and VICKERY and PUCHER (**12**)], and there have been a few more recent investigations, notably by WOLF (**16, 17**). The fundamental observations have been repeatedly confirmed, and many additional facts have been recorded; it cannot be claimed, however, that a great deal of light has been shed upon the chemical mechanisms involved, despite the manifest and widely recognized importance of the problem. Nevertheless, the development in recent years of reasonably satisfactory and accurate methods for the analysis of plant tissues and, more especially, the identification of MAYER's alleged isomer of malic acid (the so-called "crassulacean malic acid") as isocitric acid (**5, 10, 11**) have led to the hope that renewed study might reveal helpful information. In this series of papers, accordingly, the results of analytical investigations, chiefly of the leaves and stems of *Bryophyllum calycinum*,

are to be described; the present paper deals with the changes in composition that occur during normal growth. The experiments were carried out in order to see to what extent the changes in chemical composition of a plant which has a pronounced diurnal variation in organic acidity may differ from those of a plant, such as tobacco, which does not display this phenomenon.

Preparation of samples

Leaves picked from Bryophyllum plants derived originally from a single specimen were placed on moist sand in the greenhouse on September 1, 1940, and, 7 weeks later, the plantlets that had formed on the margins were separated and transplanted. On January 2, 1941, 30 young plants were again transplanted into sand in individual 1-gallon crocks and were thereafter flushed three times a week with a nutrient solution of the composition 0.001 M KH_2PO_4, 0.00225 M $Ca(NO_3)_2$, 0.0041 M $MgSO_4$, 0.0021 M KNO_3, 0.0043 M $Mg(NO_3)_2$ and which contained, in addition, 1 mg. per liter each of boron, manganese, and iron. The solution was adjusted to pH 6.0 by the addition of 0.4 ml. of 1.0 N sulphuric acid per liter. On March 27, 20 plants of uniform height and leaf size were selected and divided into four similar groups of five each. One of these groups was taken for the initial sample of the growth experiment (recorded as zero time).

The samples were harvested at 32, 62, and 95 days, on dates selected according to the weather, a bright cloudless day being chosen. The leaves were broken off at the junction with the petiole, the operation invariably being begun between 12:05 and 12:15 P.M. (standard time) in order to provide as great a degree of similarity as possible with respect to the stage in the deacidification process that had been reached in the different samples. The thick, mature, dark green basal leaves were kept separate from the thinner, smaller and lighter green upper leaves. The separation into groups was arbitrary and largely subjective but permitted comparisons to be made between the composition of mature and of relatively immature leaf tissue when the data are expressed in concentration units. Counts and fresh weights were taken and the tissue was then immediately dried in an oven at 80° C. in rapidly circulating air.

After removing the leaves, the stalk was cut at the sand level and the fresh weight, including the petioles, was determined. The tissue was then cut transversely into thin slices and likewise dried. The total time required until the tissues were placed in the oven was approximately 1 hour.

A similar procedure was followed on each of the sampling dates. However, it was necessary to discard 1 plant each from the 32- and the 62-day samples because it was less well developed than the others and had lost a few leaves; 2 plants of the 95-day sample were discarded for the same reasons. After being dried, all samples of tissue were weighed (crude dry weight) before being broken up, and were then powdered in a Wiley mill and subsequently preserved in closed containers. The fundamental data on the counts and weights are assembled in table I. All analytical determinations were

made upon these samples, the results being obtained as percentages of the crude dry weight and then computed in terms of grams, or milliequivalents in the case of organic acids, per single plant, or in grams per kilogram of original fresh tissue weight (*i.e.*, in concentration units), by means of factors obtained from the fundamental data. The one method of calculation provided a means of comparison between the composition of single plants at different stages of growth, the other permitted comparisons upon a concentration basis as growth progressed.

TABLE I

FUNDAMENTAL DATA ON COUNTS AND WEIGHTS OF SAMPLES OF *Bryophyllum calycinum* PLANTS

Growth period (days)	0	32	62	95
Sampling date (1941)	3/27	4/28	5/28	6/30
Number of plants	5	4	4	3
Number of mature leaves	33	68	124	149
Number of young leaves	69	139	187	140
Total number of leaves	102	207	311	289
Total number of petioles	57	65	85	60
Average length of stem (cm.)	17	39	67	90
Crude dry weight of mature leaves (gm.)	19.2	44.9	75.0	110.9
Crude dry weight of young leaves (gm.)	10.6	39.5	78.9	57.7
Crude dry weight of stems and petioles (gm.)	6.4	24.8	58.9	74.2
Crude dry weight of roots (gm.)	4.9	13.4	23.8	23.8
Fresh weight of mature leaves (gm./plant)	52.9	122.8	208.4	366.0
Fresh weight of young leaves (gm./plant)	25.4	110.2	203.2	181.9
Total fresh weight of leaves (gm./plant)	78.3	233.0	411.6	547.9
Fresh weight of stems and petioles (gm./plant)	14.1	77.1	181.6	267.7

Analytical methods

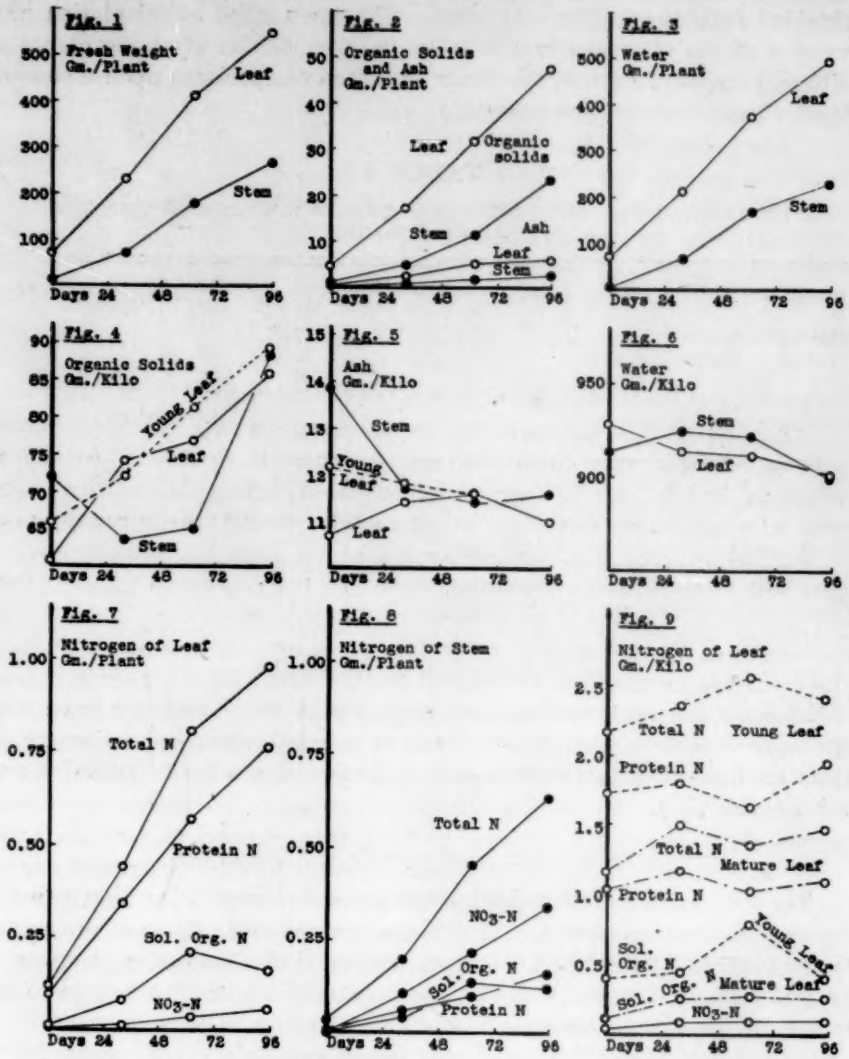
Many of the analytical methods employed in this laboratory have been specially developed and are described in journal papers. A summary of these methods, with full references, is to be found in a recent bulletin from this Station (**15**).

Growth in terms of organic solids and ash

The data for the fresh weight, the organic and inorganic solids, and the water content of both leaf and stem tissue are plotted in terms of grams per single plant in figures 1 to 3. During the period of observation, the growth rate of both the leaves and stems was essentially linear with respect to increase in fresh weight although that of the stems appears to have been slightly accelerated with respect to the rate of accumulation of organic solids as the plants increased in size.

A comparison of the rate of growth of the Bryophyllum plant with that of the tobacco plant is of interest. Data obtained during the season 1933 (**13**) for tobacco plants grown in the field under conditions of commercial production indicated that, 26 days from transplanting, the plants had become well established. At this point they were just passing into a period about 35 days long during which they grew at an essentially linear rate, and

the leaves were found to increase in fresh weight at an average rate of 15 grams per day, the stalks at about 16.6 grams per day. These figures are to be contrasted with an average rate of 5.7 grams per day for the leaves and 2.7 grams per day for the stems of the Bryophyllum plant over the 95-day



period of the present experiment. Clearly the tobacco plant grows much more rapidly than the Bryophyllum plant when compared at presumably analogous points in the course of their respective development.

Figures 4, 5, and 6 show the organic solids, the ash, and the water content of the leaf and stem tissue in concentration units. Although the data for the organic solids of the total leaf tissue indicate a somewhat irregular

increase in concentration, those for the young upper leaves (broken line) follow a nearly linear course. In the stem, on the other hand, the concentration of organic solids decreased at first and then increased rapidly.

The concentration of the ash constituents in the young leaves was higher than the average for the whole mass of leaf tissue in the plants at the start, but the difference became inappreciable as growth progressed. The concentration of ash constituents in the stem decreased rapidly at first, but then levelled off and remained essentially constant after 32 days of growth.

Figure 6 shows that the water content of both leaf and stem tissue remained between the limits 90% and 93% of the fresh weight throughout the experiment. The data for the leaf suggest a small continuous decrease in water content with age whereas the stem increased slightly at first and then decreased. Although this plant is classified as a "succulent," the water content of the leaves does not differ greatly from that of tobacco. Leaves from young growing tobacco plants contain from 89% to 90% of water.

Growth in terms of nitrogenous components

The rate of accumulation of total nitrogen, protein nitrogen, soluble organic nitrogen, and nitrate nitrogen is shown in figure 7. Growth, as measured by total nitrogen, gives a curve that is slightly concave to the time axis although, as measured by organic solids (fig. 2), the curve is linear. It may be inferred that, toward the end of the period of observation, the rate of synthesis of organic substances free from nitrogen gradually increased relatively to the rate of synthesis of nitrogenous organic substances. Nevertheless, the rate of synthesis of protein in the leaf is shown by a curve that departs very little from linearity. The curve for the soluble organic nitrogen (*i.e.*, soluble nitrogen exclusive of nitrate), however, indicates that the rate of accumulation of this group of substances was not maintained in the last interval between observations in spite of the fact that the storage of nitrate nitrogen in the tissue increased regularly. The plants were clearly living under conditions of liberal, if not luxury, supply of nitrate nitrogen, and the alteration in the rate of accumulation of soluble nitrogenous organic substances reflects an alteration in the general course of the metabolism as the plants matured. As is shown in figure 9, the fluctuations in the concentration of the soluble nitrogenous substances in the young upper leaves go far to account for this change; the concentration in the lower older leaves remained essentially constant.

The data in figure 8, showing the rate of accumulation of nitrogen in the stem, are plotted on the same scale as those of figure 7. Although the total nitrogen of the stem increased almost linearly, at each stage of observation slightly more than one-half of the nitrogen was present as nitrate, and only about one-quarter as protein. Both nitrate and protein nitrogen accumulated at a steady rate. However, the soluble organic nitrogenous substances decreased in the stem during the last period of observation as they did in the leaf, again reflecting the change in the general course of the nitrogen metabo-

lism as the plants approached maturity. The large quantity of nitrate suggests a storage function for the stem tissue. Analogous observations have been made for both tomato (**2**) and tobacco (**13**) plants, and the phenomenon is doubtless common.

The concentration of the total nitrogen and of the protein nitrogen in both the young and the mature leaves is plotted in figure 9, together with the concentration of the soluble organic nitrogen. There is a marked contrast between the young and the older leaves; the young leaves were much richer both in nitrogen and in protein and the curves for the latter component, although somewhat irregular, run parallel to each other for most of the period studied. The curve for the concentration of organic soluble nitrogen, like that of the total quantity per plant (fig. 7), reveals a moderate increase followed by a decrease in the last period of observation. The nitrate nitrogen in the whole leaf tissue increased in concentration.

The relative decrease in soluble organic nitrogenous substances coupled with an increase in the protein nitrogen in the plants 95 days old as compared with those 62 days old is of considerable interest. The change is evident in the data for absolute quantities per plant as well as in those expressed on a concentration basis. Examination of the detailed data for the young and for the more mature leaves shows that, during the last interval between observations, the concentration of protein nitrogen in the mature leaves increased very little while that in the young leaves increased materially. However, the concentration of soluble organic nitrogenous substances in the mature leaves remained constant while that in the young leaves decreased. Thus, in the mature leaves, there was a nearly constant relationship between the concentrations of soluble nitrogenous substances and the protein, although both increased in absolute amount per plant. In the young leaves, on the other hand, an increase in the concentration of the protein was associated with a marked decrease in the concentration of the soluble nitrogenous substances.

The careful review by PETRIE (**9**) of the literature of protein synthesis in the plant suggests that protein synthesis is correlated with the level of amino acids in the cell solution but that the relationship is not a simple one; other factors, especially the respiration rate and, accordingly, the rate of liberation of energy, play a part. The present data are not in conflict with Petrie's view of a "drifting steady state" with respect to the relationship between the protein and the amino acids of the plant cells. A reciprocal relationship between the concentration of the soluble nitrogen and of the protein nitrogen is evident in the young leaves although not in the more mature ones. This suggests that the high concentration of soluble nitrogen at 62 days may have been a factor in the relative increase in the concentration of protein nitrogen observed later in the young leaves of the plants.

Figure 10 shows the changes in the concentration of the nitrogenous substances in the stem and should be compared with figure 8 which shows the absolute quantities. There was a sharp increase in the concentration of the

total nitrogen at the start, and much of this arose from the increase in nitrate concentration. Subsequently, the concentration of the nitrate diminished slightly and then remained nearly constant. Nitrate nitrogen made up about one-half of the total nitrogen of the stem throughout the period studied. A high concentration of protein nitrogen is associated with a low concentration of soluble nitrogen in the youngest plants, but this situation is reversed in those 62 days old. In the 95-day-old plants, the concentration of protein nitrogen had increased slightly while that of the soluble nitrogen diminished materially. The reciprocal relationships in the stem are thus analogous to those observed in the young leaves.

Growth in terms of organic acids

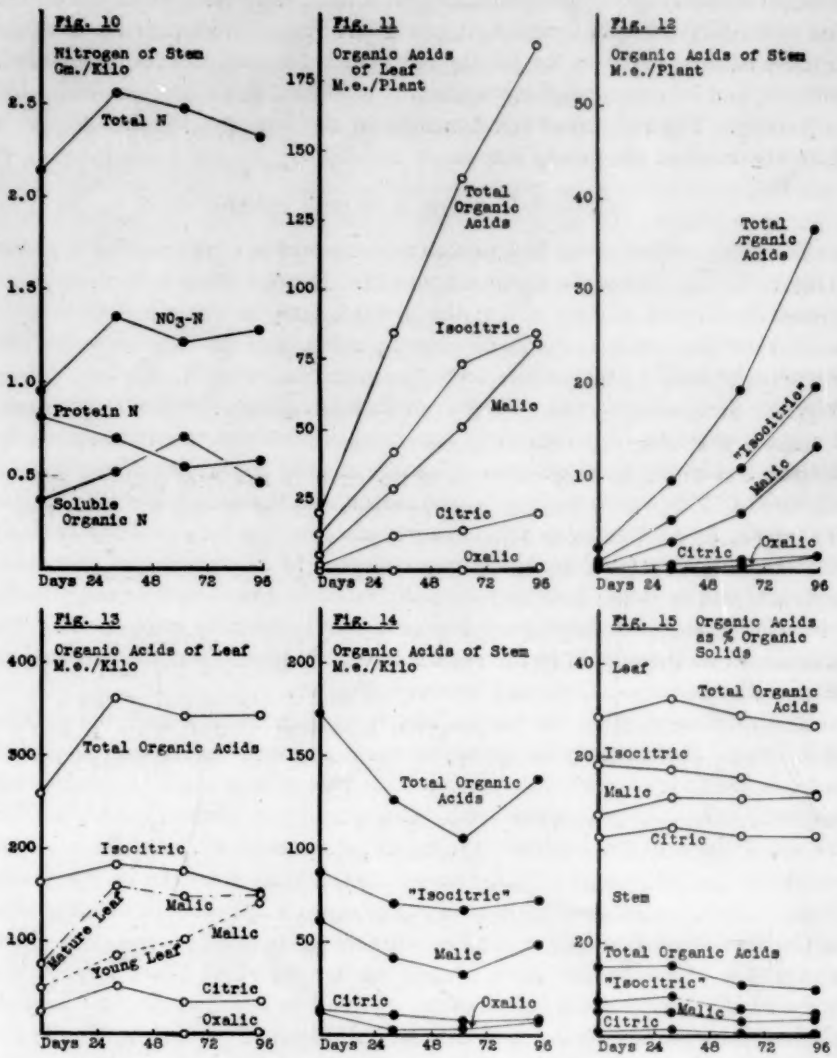
The composition of the leaf tissues with respect to organic acids is shown in figure 11 and that of the stem in figure 12. Bryophyllum leaves are extraordinarily rich in organic acids; the present samples contained from 26% to 32% of the organic solids as organic acids, and these substances are, accordingly, major components from the quantitative point of view. However, the diurnal variation in the organic acids present makes it necessary to specify the time of collection before quantitative statements have precise meaning. During the night, the quantity of acid, especially of malic acid, increases but this process is reversed as soon as the leaves are illuminated so that the level of organic acids has greatly decreased by midday or early afternoon. The present samples were collected at a few minutes past noon on bright sunny days; had they been collected in the early morning, malic acid would doubtless have been the predominating acid component. The smoothness of the curves in figures 11 and 12 suggests that the precautions taken with respect to sampling time were effective.

The total acidity of the leaves, like the organic solids and the protein nitrogen, follows a nearly linear course throughout the experimental period, and this is also true of the malic acid. The rate of accumulation of isocitric acid declined as the plants matured. Citric acid was present in only moderate quantities and the increase during growth was small. Oxalic acid was present in the leaf tissue only in traces, the total amount even in the 95-day plants being so small as to be scarcely apparent on the scale of the diagram.

Organic acids make up a smaller relative proportion of the stem tissue than of the leaf. Figure 12 is plotted on a scale of $3\frac{1}{3}$ times larger than figure 11 in order to show the changes. It will be recalled that the organic solids of the stem amounted to about one-half those of the leaves (fig. 2) but that the stems of even the oldest plants contained less than one-fifth as much organic acid as the leaves. This is the reverse of the situation observed in the rhubarb plant where the petiole was found to be far richer in organic acids (**14**) than the blade.

The general picture of the rate of accumulation of organic acids in the stem is, however, not unlike that in the leaves. The total organic acids increased along a straight line curve for 62 days, but the curve then turned

upwards as the relative quantity of organic solids increased in the more mature stems. The curves for "isocitric" acid and malic acid conform. Neither citric nor oxalic acid was present in more than traces in the stem tissue.



Some discussion is necessary of the meaning of the term "isocitric acid" as used in the analyses of these tissues. It has been shown that by far the greater part of the difference between the total organic acids and the sum of the malic, citric, and oxalic acids in Bryophyllum leaves consists of isocitric acid (**10**). Accordingly, this difference, which in previous papers from this laboratory is denoted as "unknown organic acid," may, in the

particular case of the leaves of Bryophyllum, be used as an approximate measure of the isocitric acid content. The error with this tissue is certainly not important. But we have obtained, as yet, no direct evidence that isocitric acid is present in the stems of this plant. It seems a reasonable assumption, however, that the qualitative composition of the stem will not differ greatly from that of the leaf and, accordingly, the proportion of unknown organic acids in the stem has been provisionally denoted as "isocitric acid." Analytical methods for isocitric acid have been developed during the past two years in other laboratories (**5, 8**). These methods, however, depend on the use of specific enzymes, and their application to the analysis of plant tissues has not yet been made in this laboratory.

Figure 13 shows the organic acids of the leaf tissue expressed in concentration units. During the first 32 days of growth, there was a marked increase in the concentration of the total acids much of which arose from the increase in malic acid; subsequently the concentration diminished moderately. The behavior of malic acid in the mature and in the young leaves was different. The young leaves showed a steady increase in malic acid throughout the experimental period, but the mature leaves increased in malic acid only in the first interval between observations.

The behavior of the concentration of the acids in the stem tissue is shown in figure 14, which is plotted on a scale twice that of figure 13. The total acids decreased for the first 62 days and then increased, and both malic acid and isocitric acid shared in the change. The small concentrations of citric and oxalic acids present altered very little.

Figure 15 shows the organic acids of both leaf and stem tissue as percentages of the organic solids. Isocitric acid is the predominant organic acid and was present to the extent of 18% of the organic solids of the leaves of the youngest plants but the proportion decreased as the plants matured. Young plants are thus obviously to be preferred for the preparation of isocitric acid in quantity. With such material, the relative proportion of isocitric to citric acid is greatest, a circumstance favorable for the separation by chemical means, and greater yields would be anticipated. It would also be more economical of time to work with young plants.

The data for the concentration of the acids in the organic solids of the stem are plotted in the lower part of figure 15. As has already been pointed out, this tissue is relatively low in organic acids and the acidity diminishes with increasing age. The stems of the young plants contained 15% of the organic solids as organic acids and this proportion dropped to 10%. "Isocitric acid" made up about 8% of the solids of the young stems and 5% of the oldest. The proportion of malic acid was somewhat less in each case.

Growth in terms of carbohydrates

Figure 16 shows the rate of increase in total and unfermentable carbohydrates in the leaf tissue, the data representing the reduction measured with the Shaeffer-Somogyi sugar reagent, and being expressed in terms of

glucose. The leaf tissue is moderately rich in sugar, the composition, expressed as percentage of the organic solids, ranging from 8.6% to 6.8% in the mature leaves, and from 3.7% to 4.9% in the young leaves. The present figures represent, of course, only the condition at the time of collection of the samples with respect to a component that fluctuates within fairly broad limits during the day. However, the rate of accumulation of total carbohydrate was essentially linear over the period of observation, suggesting that the samples were taken at comparable stages in this metabolic process.

The component designated unfermentable carbohydrate represents a titration for sugar carried out after treatment of the extract from the leaf with yeast. Clearly, a substantial part of the total carbohydrate consists of substances that are not removed by this treatment, and it is well known that components of similar behavior are commonly found in leaf tissues. The rate at which this type of carbohydrate accumulated seemed to increase moderately as the plants developed.

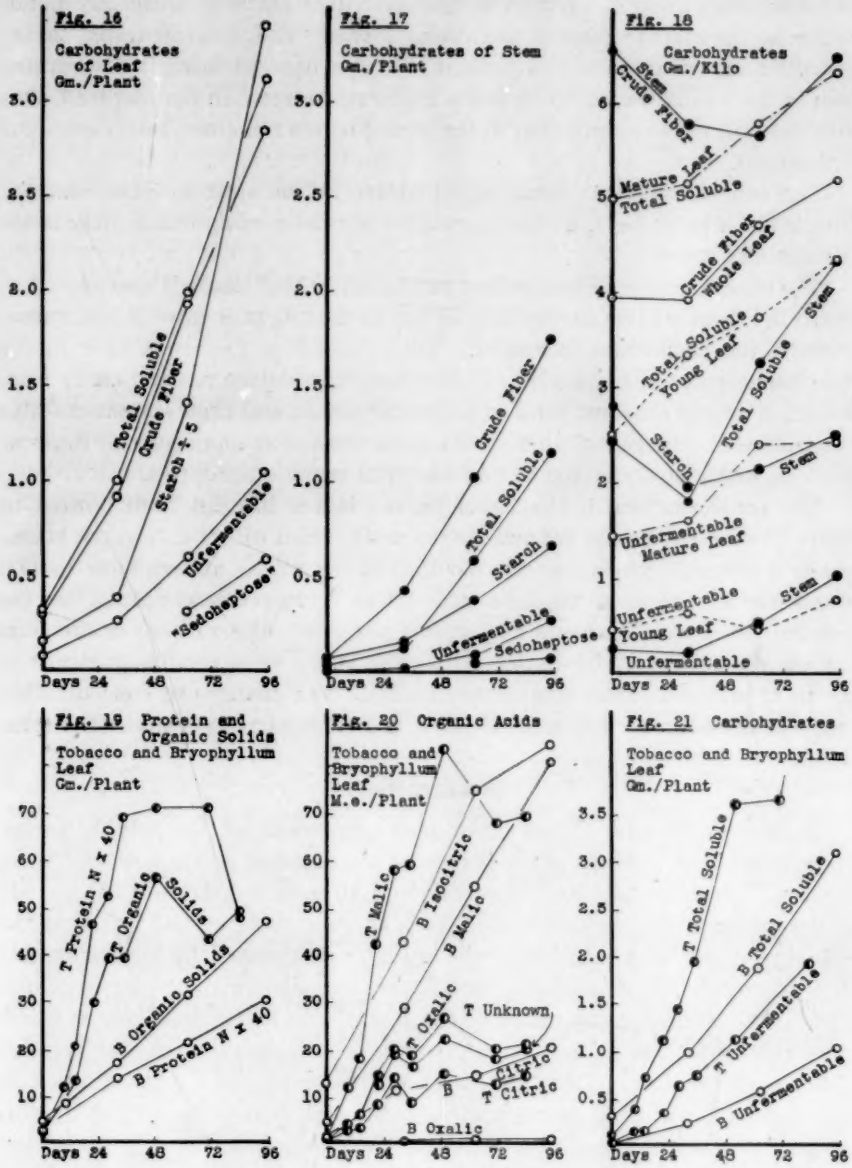
The quantities of crude fiber, determined by the conventional analytical method, are also shown in figure 16. This material doubtless consists mainly of cellulose and the rate of accumulation serves as an approximate measure of the rate of growth of the cell walls. The curve is, within the limits of error, linear throughout the period of study. It is of interest that the quantities of crude fiber in the leaf tissue are nearly identical with the quantities of soluble carbohydrates in each sample. However, no significance should be attached to the coincidence of the curves. No such agreement was observed in the case of tobacco leaves; in these, the slopes of the analogous curves were quite different.

The chief carbohydrate component of the leaf tissue was starch. The analytical values have been divided by 5 in order to bring them within the scale of figure 16, and it is to be noted that the rate of accumulation of starch was continually accelerated as the plants matured. By far the greater part of the starch was found in the older leaves and it is clear that the rate of growth, as measured by the accumulation of starch, is a function quite different from the rates as measured, for example, by the organic solids or the protein nitrogen, both of which were essentially linear. As the plants increased in age, the leaves became increasingly efficient with respect to the synthesis, or at least the storage, of starch.

The data plotted in figure 17 shows that the stem is, in general, lower in carbohydrates than the leaves. The values for total carbohydrate range from 3% to 5% of the organic solids. Unfermentable carbohydrate was present although in relatively small proportion. The rate of accumulation was slow at first but then followed an approximately straight line curve.

The most important carbohydrate component of the stem tissue was cellulose as is shown by the curve for the crude fiber. The rate of deposition of cellulose was slow at first, but proceeded subsequently at a linear and more rapid rate. However, there was less crude fiber in the stem than in the leaves throughout the period of observation. This is in marked contrast to the

behavior of the tobacco plant, the woody stem of which soon exceeds the leaves in quantity of crude fiber present and, at maturity, contains ten or more times as much.



The stems contained only moderate quantities of starch, less indeed than of soluble carbohydrates, and the rate at which starch appeared conforms with the rates for the soluble carbohydrates. There would appear to be little connection, therefore, between starch synthesis in the stem and that in the leaves.

The concentration of the carbohydrate components in terms of fresh weight is shown in figure 18. In the mature leaves, the total carbohydrates increased moderately with age, but, in the young leaves, the rate of increase was somewhat greater. However, the concentration was invariably much higher in the mature than in the young leaves. The unfermentable carbohydrate components were also present in higher concentration in the mature than in the young leaves; there was a moderate increase in the mature leaves with age, but the concentration in the young leaves remained fairly constant throughout.

The concentration of total carbohydrate in the stem dropped sharply during the first 32 days, but subsequently increased to approximately twice the minimal value.

The concentration of crude fiber in the entire leaf tissue is also shown in figure 18; there was no change during the first 32 days of growth but, subsequently, the proportion increased. The behavior of the crude fiber in the stem tissue was quite different; it decreased in relative proportion at first, remained nearly constant for a considerable period and then increased. The concentration changes of fiber in the stem were thus analogous to those of the total organic solids (fig. 4) and the total organic acids (fig. 14).

The concentration of the starch in the leaves has not been plotted in figure 18 since the values are outside the scale of this diagram. In the young leaves it ranged from 8.8 grams per kilo in the plants at zero time to 17.0 grams per kilo in those 95 days later. The corresponding figures for the mature leaves were 11.1 and 24.7 grams per kilo. The values for the stem are shown, however, and indicate that the stems were relatively richer in starch at zero time than they later became. The changes in concentration conform moderately well with those of the total carbohydrate in the same tissue.

Sedoheptose

In figures 16 and 17, an additional component of the soluble carbohydrate fraction is shown at the bottom of the diagram and is marked "sedoheptose." Sedoheptose was discovered by LaForge and Hudson (**6**) in 1917 in the leaves of *Sedum spectabile*. It is characterized by the fact that the reducing power of a solution of the sugar is diminished by approximately 80% of its original value when the solution is heated with 1% hydrochloric acid. The change is the result of the loss of one molecule of water which gives rise to the formation of an anhydride or sedosan; the reaction appears to be an equilibrium. Bennet-Clark (**1**) has employed a method based on this change in reducing power on treatment with acid to detect sedoheptose in the leaves of succulent plants, among them *Bryophyllum calycinum*, and has further noted, in the case of *Sedum praealtum*, that the sugar component which has this property undergoes wide diurnal variations in amount, these variations being reciprocal with those of the acidity. Wolf (**16**), however, was unable to confirm this in the case of *Bryophyllum calycinum;* rather, he demonstrated reciprocal variation of the fermentable carbohydrates and

of the starch as against the changes in the acidity. Nevertheless, he secured evidence of the presence of sedoheptose in Bryophyllum leaves by the isolation of a phenylosazone that corresponded closely in properties to that described by LAFORGE and HUDSON.

A brief study of the reducing power of extracts from Bryophyllum leaves confirmed the presence of a carbohydrate which was diminished in reducing power when the extract was boiled with dilute acid. These analyses were carried out on extracts that had not been treated with yeast; otherwise lower and erratic results were obtained. Experiments in which sulphuric acid of concentrations in the range 0.2 to 1.0 N were used showed that a concentration of 0.35 N was most satisfactory for the formation of the anhydride ring. With this reagent, a maximal diminution of the reducing power was secured after the solution had been boiled for 30 minutes. No further loss occurred after the solution had been boiled for 30 additional minutes. Weaker acid reagents required longer times while a reagent as strong as 1.0 N gave smaller diminutions probably because of the hydrolysis of complex carbohydrates.

The diminution of the reducing power, expressed as glucose, is plotted in figure 16 as "sedoheptose" in spite of the fact that it is known that the anhydride formation with pure sedoheptose is incomplete. LAFORGE and HUDSON state that the equilibrium occurs when 80% of the sugar has been converted, while BENNET-CLARK observed 75%. Furthermore, the relationship between the sugar reagent standardized in terms of glucose and the quantity of sedoheptose reduced is unknown. Because of these two uncertainties, it seemed best to plot the actual quantities of reagent reduced, in terms of glucose, and to postpone the interpretation in terms of the quantities of sedoheptose involved.

With these restrictions of the meaning of the term defined, "sedoheptose" was observed in significant amounts only in the mature leaves of the 62- and 95-day plants. In these, it made up a substantial part of the unfermentable carbohydrate, almost certainly a larger part than is suggested by the relative positions of the curves drawn in the figures. The more mature leaves of the youngest plants gave evidence of only a trace of this component, while the young leaves of all save the 95-day plants gave either an increase in reduction when the extracts were heated with acid or no change. The stems of the 62- and 95-day plants also showed evidence of a trace of "sedoheptose" but, if any were present in the stems of the 0- and 32-day plants, it was not detected since the reduction was increased slightly by the treatment with acid. Clearly, therefore, "sedoheptose" is a component that could be demonstrated only in the mature leaves of fairly old plants and, possibly, in the stems of the same plants.

Composition of Bryophyllum tissues

Table II shows estimates of the quantitative composition of the leaf and stem tissue of these samples of Bryophyllum plants. The data for protein

TABLE II

COMPOSITION OF BRYOPHYLLUM PLANTS
Figures not otherwise designated are grams per single plant.

	Leaf				Stem			
Growth period (days)	0	32	62	95	0	32	62	95
Protein (N × 6.25)	0.619	2.181	3.456	4.756	0.075	0.337	0.625	0.994
Soluble nitrogenous components (N × 10)	0.150	0.780	2.13	1.62	0.050	0.390	1.29	1.22
Starch	0.810	2.07	7.02	12.15	0.040	0.153	0.393	0.668
Crude fiber	0.310	0.913	1.94	2.85	0.093	0.447	1.03	1.75
Ether extract	0.132	0.534	0.992	1.48	0.015	0.068	0.147	0.373
Soluble carbohydrate	0.331	1.003	1.970	3.116	0.035	0.140	0.598	1.165
Isocitric acid	0.828	2.736	4.685	5.398	0.079	0.349	0.707	1.272
Malic acid	0.361	1.980	3.465	5.466	0.057	0.213	0.408	0.886
Citric acid	0.128	0.777	0.930	1.323	0.012	0.055	0.080	0.120
Oxalic acid	0.002	0.006	0.033	0.063	0.007	0.014	0.034	0.082
Total known organic solids (estimated)	3.671	12.98	26.62	38.22	0.463	2.166	5.312	8.530
Total organic solids (determined)	4.80	17.25	31.83	47.44	1.02	4.90	11.82	23.55
Unknown organic solids	1.18	4.27	5.21	9.22	0.557	2.73	6.51	15.02
Unknown organic solids as percentage of total organic solids	24.6	24.7	16.4	19.4	54.6	55.7	55.1	63.8
Inorganic solids	0.84	2.66	4.77	6.01	0.196	0.907	2.075	3.10

are computed from the protein nitrogen by multiplication by the conventional factor 6.25, a procedure that is admissible in the absence of information on the actual nitrogen content of the tissue protein. The estimate of the quantities of soluble nitrogenous components is made by multiplying the data for non-protein soluble nitrogen, exclusive of nitrate, by the factor 10. This is doubtless an overestimate since the average nitrogen content of the substances in this group, presumably amino acids, amides, polypeptides, and basic substances such as choline, purines, and basic amino acids is greater than 10%. However, it has been found, in other samples of Bryophyllum leaves, that from 35% to 40% of the soluble nitrogen is normally present as amino nitrogen; thus a considerable part of the substances concerned probably does consist of amino acids and the factor 10 can be used for these without grave error.

Table II also gives data for the ether extract of the samples. This was obtained by the conventional procedure and represents chlorophyll, lipid pigments, and other fat-solvent soluble material; probably only a small part of it consists of true fat.

The quantities of organic acids were computed from the analyses in terms of milliequivalents by the use of the appropriate factors.

The sums of the estimates are shown, together with the determined quantities of organic solids; the difference between these thus represents that part of the organic material concerning which no qualitative information is available. In the leaf tissue, this unknown part amounts to from one-quarter to one-sixth of the organic solids. In the stem tissue, on the other hand, the unknown part makes up appreciably more than one-half of the total, and suggests that a number of major components await detection and determination. Nothing but speculation can be offered concerning these unknown components in either leaf or stem tissues. However, such substances as glucuronides of the pectin type are probably present in the leaf and complex carbohydrates such as hemicellulose in the stem. Clearly, much remains to be learned regarding the qualitative composition of the tissues of this plant.

Comparison of Bryophyllum with tobacco

In figures 19, 20, and 21 are shown data taken from a study of the rate of growth of the tobacco plant (**13**) plotted together with a few of the more important items of the present data on the Bryophyllum plant in order to afford a comparison of the behavior of the two species. Figure 19 shows the organic solids and the protein nitrogen of the leaf tissues, these being selected as perhaps the most clearly illustrative of the relative rates of growth. The protein nitrogen values have been multiplied by 40 in order to obtain curves that can be plotted on the same diagram with the solids. Zero time for the tobacco plants is arbitrarily taken as the point at which the composition with respect to nitrogenous components was essentially identical with that of the youngest Bryophyllum plants. This occurred 26 days after the tobacco seedlings had been set in the field when the plants had become well established

and were just beginning to grow at a rate which followed a nearly linear course for the next four or five weeks. It is assumed that, during this period, the plants were passing through a phase of growth analogous to that of the present series of Bryophyllum plants. At all events, this was the purely vegetative stage in the growth period of the tobacco plants. The first samples of inflorescence tissue, mainly flower buds, were collected from the tobacco plants at 61 days from transplanting (*i.e.*, at 35 days on the scale of figures 19 to 21) and subsequent samples contained increasing quantities of flowers and developing seed pods. The change in the course of the metabolism, as the plants passed into the reproductive phase, is illustrated on the curve for the leaf protein which flattened out and then fell as protein was withdrawn from the leaves during the ripening of the seeds. There was also a loss of organic solids from the leaves. The main point of interest in the present comparison is, however, the contrast between the relative rates at which leaf protein and leaf organic solids were formed during the first 40 days of growth of the tobacco plant and their behavior in the Bryophyllum plant. Clearly, the tobacco leaf is considerably richer in protein, in relation to the organic solids, than is the Bryophyllum leaf, while the rate of growth, as measured by the rate of accumulation of the leaf protein, is about four times as rapid. The rate of growth, as measured by the organic solids of the leaf, is about twice as great.

Figure 20 shows the composition of the leaves of the two species with respect to organic acids. In the tobacco leaf, malic acid is by far the predominating acid component, and the curve follows in fairly close detail that for the organic solids in figure 19. Oxalic acid is next in relative importance as a component, and the "unknown" organic acids are present in closely similar amounts. The curve for the "unknown" acids of the tobacco leaf should be compared with that for isocitric acid in the Bryophyllum leaf, the main acid component of this species. Both curves depend upon analytical data obtained in the same way, the difference being that the "unknown" acids of the Bryophyllum leaf have been shown to be comprised largely of isocitric acid. Whether or not isocitric acid occurs in tobacco leaf has not been demonstrated. However, observations on the organic acid esters of high boiling point obtained from tobacco leaf tissue suggest that isocitric acid can be present only in traces if at all.

Citric acid is a minor component of both the tobacco leaf and the Bryophyllum leaf, and the curves for the rate of accumulation in the two species are closely similar; in fact this is the only point in which the two are closely alike.

The curve for malic acid in the Bryophyllum leaf represents the composition with respect to this component at noon on sunny days. Had the samples been collected earlier in the day, the curve would, doubtless, have been displaced vertically upwards and might also have had a somewhat different slope. Nevertheless, the rate of accumulation of malic acid in this plant, as a function of age and with this restriction on the time of collection, is clearly less than it is in the tobacco plant.

The curves for the total organic acidity of the two species are not plotted in figure 20; they show, however, that the tobacco plant accumulates organic acids in the leaves, during the approximately 40-day period of its maximal growth rate, somewhat more rapidly than the Bryophyllum plant. However, the check that is placed on the development of the tobacco leaf by the onset of the reproductive phase brings the synthesis of organic acids to a stop, while accumulation of acids in the Bryophyllum leaves continues as is shown in figure 11. Nevertheless, if observations on Bryophyllum leaves were made in the early morning, there is little doubt that the rate of accumulation of total acids would closely approach that of the tobacco leaf.

The qualitative composition and, with the exception of citric acid, the relative rates of synthesis of the organic acids in the two species are entirely unlike. The differences with respect to oxalic acid and isocitric acid are perhaps the most striking. Oxalic acid is an important component of tobacco leaf tissue but is present in Bryophyllum leaves only in traces. The reverse is true for isocitric acid. The metabolic systems that lead to the synthesis of organic acids in the two plants are thus entirely unlike; although there are, doubtless, certain features common to the two, since malic and citric acids are components of both, the details of the chemical mechanisms in which organic acids share are manifestly widely different.

Figure 21 shows the relative rates of accumulation of soluble carbohydrates in tobacco and Bryophyllum leaves. Although the type of tobacco plant described (the so-called Connecticut shade-grown tobacco) is characterized by a somewhat low level of carbohydrate content, particularly of starch, it is obviously a species that synthesizes soluble sugars in the leaves more rapidly and in larger quantities than the Bryophyllum plant. Much of the unfermentable carbohydrate of the mature Bryophyllum leaf is doubtless sedoheptose but this is certainly not true of the tobacco leaf, although unfermentable carbohydrates make up a large part of the soluble sugars in this species. Thus, both qualitatively and quantitatively, the composition of the two kinds of leaves is widely dissimilar.

Summary

The composition of the leaf and stem tissue of *Bryophyllum calycinum* plants, harvested at noon on sunny days at intervals over a period of 95 days, has been determined in order to obtain fundamental data upon the rate of growth of a plant which is characterized by a pronounced diurnal variation in organic acid content. The composition is recorded in terms of grams per plant. The rate of accumulation of the fresh weight and of many of the components, in particular the organic solids, the ash, the water, the protein, the nitrate nitrogen, the soluble carbohydrates, the crude fiber (cellulose), the total organic acids, and the malic acid followed essentially straight line curves throughout the period of observation. On the other hand, the total nitrogen followed a curve somewhat concave to the time axis, as was true also of the isocitric acid and citric acid, suggesting a gradual slowing of the rate

of accumulation of these components, while the starch of the leaves, by far the most plentiful known component of this tissue, followed a curve markedly convex to the time axis indicating an increase in relative capacity for the storage of starch as the leaves matured.

The data have also been computed in concentration units, namely in grams per kilo of fresh weight of the tissues. These curves show a moderate degree of irregularity in the relative concentrations of the various components at different stages of growth.

Comparison of the data with similar results for the tobacco plant, at an analogous period in the life cycle, showed marked differences in the rate of accumulation of most of the components. The tobacco plant grows much faster as measured by almost all of the criteria. The most important exception is in the rate of accumulation of organic acidity; isocitric acid is formed almost as rapidly by the Bryophyllum plant as malic acid is by the tobacco plant, and citric acid is formed at almost equal rates in the two species. On the other hand, oxalic acid, which is a major organic acid component of the tobacco plant, is present only in traces in Bryophyllum.

Isocitric acid is the predominating organic acid component of Bryophyllum leaf tissue and is present in young leaves to the extent of about 18% of the organic solids; such tissue is accordingly valuable for the preparation of this rare acid in quantity.

We are indebted to the DEPARTMENT OF ANALYTICAL CHEMISTRY of this Station for the determinations of crude fiber and ether extract in these samples.

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SOME MOLD-INDUCED CHANGES IN SHELLED CORN[1]

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(WITH FOUR FIGURES)

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Introduction

Shelled corn which molded during storage was found to be high in fat acidity and moisture percentage and low in bushel weight (10). Because many different fungi were associated with the molded condition of the corn, the extent to which the individual fungi contributed to these changes was of interest. Laboratory studies were conducted by infesting sterile corn with pure cultures of different fungi which were obtained from the naturally molded shelled corn found in the storage bins (10). Determinations were made on the laboratory-molded corn for changes in organic matter, water-soluble and -insoluble nitrogen, fat acidity, pH and water content. These same fungi were also cultured on Czapek-Dox medium to determine their carbohydrate-utilizing and acid-producing capabilities on this medium in comparison with corn.

Materials and methods

The 9 fungi used were: *Aspergillus flavus* Link, *A. candidus* Link, *A. niger* van Tieghem, *A. amstelodami* (Mangin) Thom and Church, *Penicillium palitans* Westling, *P. chrysogenum* Thom, and cultures I and II *P. rugulosum* Thom and *Mucor racemosus* Fresenius (these cultures differed in color and in growth rates on agar media). Subcultures of each of these were prepared in quadruplicate on sterile corn as follows: Lots of 150 gm. of Iowa hybrid 939 corn with approximately 15% moisture were placed in each of a number of 500-ml. Erlenmeyer flasks which were plugged with cotton and steamed at 15 lbs. pressure for 45 minutes on two consecutive days. After the final steaming, and while the flasks and corn were still hot, measured quantities of hot, sterilized, distilled water were added aseptically to the corn to bring it to approximately 32% moisture content. Several days at room temperature were then allowed for the corn to absorb the added moisture. This process was facilitated by frequently shaking and rotating the corn within each flask. The corn was then carefully infested with dry spores of each of the fungi except *Mucor racemosus*. The spores of the other fungi were taken from old sporulating cultures on the flattened end of a nichrome transfer-needle and carried to the mouth of the proper flask at which position they were showered. The spores of *M. racemosus* were prepared in aqueous suspension and distributed by drops in amounts of 1 ml. per flask. The con-

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tents of each flask were then mixed by hand-rotating the flask for about 1 minute and this was repeated several times over the succeeding days. The flasks of corn were then set aside in the laboratory for periods up to 4 weeks for the fungi to grow. Controls without mold infestation were also included in this test.

At the different analysis periods, the moldy corn and the mold-free controls were emptied on pieces of cheesecloth from each of the quadruplicate flasks and dried for several days at the temperature of the laboratory with the aid of an electric fan. Further drying was done at 70° C. over a 4–5-day period in an electrically heated oven, after which the material was ground for analysis to pass a 200-mesh sieve. The moisture content of the ground material was determined with a vacuum oven at 70° C. Fat acidities were determined by the method of ZELENY and COLEMAN (**14**). Water-soluble nitrogen was determined on 100 ml. of aqueous extract from 2 gm. of the material (water-free basis). This extract was prepared by shaking the material in water for 8 hours at the temperature of the laboratory. Water-insoluble nitrogen was taken as the difference between the total nitrogen in the unextracted moldy corn and its water-soluble nitrogen content. Determinations of pH were made with a Coleman electrometer on a suspension of 1.0 gm. ground material in 10 ml. of boiled, distilled water. The water content of each culture was taken as the difference between the wet and dry weights of the culture flask contents. The organic matter content of each culture was taken as the dry matter content of each flask.

Liquid culture studies of the fungi were conducted on Czapek-Dox medium. Twenty-five milliliters of this medium were placed in a number of 125-ml. Erlenmeyer flasks and sterilized at 15 lbs. steam pressure for 15 minutes. Spores of the different fungi were introduced onto this medium in a manner similar to that on corn, except that in the case of *M. racemosus* only two drops of a spore suspension were added to the medium in each flask. The fungi were allowed to grow on this medium for 1, 2, and 4 weeks at ordinary laboratory temperatures. At these periods the developed mycelium was removed from a number of cultures by decantation and filtration through pieces of washed percale cloth after which it was carefully removed from the cloth with forceps, washed in small quantities of hot, distilled water, dried at 70° C. for 3 days, and weighed in a closed weighing bottle. The filtrates from the liquid culture were increased to 100 ml. volume with distilled water, then analyzed for the remaining glucose and for other reducible substances by means of the copper method of VAN DER PLANK (**13**) and the iodometric method of HINTON and MACARA (**5**). The titrable acidity and pH of this diluted filtrate were also determined.

Experimental results

GROWTH OF THE FUNGI ON CORN

Two trials were conducted on corn to determine the organic matter, fat acidity and nitrogen changes resulting from the activity of the 9 fungi.

The first trial was divided into two parts with 4 of the 9 fungi being compared at one time and the remaining five at another time. The second trial compared all of the 9 fungi simultaneously. In the first trial, organic, matter, fat acidity, water-soluble, and -insoluble nitrogen were determined, while in the second trial water formation and pH of the molded corn were also determined. Because the second trial yielded essentially the same results as the first trial and simultaneously compared the 9 fungi and afforded the information on water formation and pH changes, its data are mainly considered here. Some pertinent data of the first trial are also presented.

Organic matter losses

As may be seen from the data of the second trial shown in table I, four of the nine fungi induced marked organic matter losses. The greatest loss (approximately 40%) was induced by *Aspergillus flavus, A. niger, Penicillium chrysogenum* I, and *P. chrysogenum* II in the 4-week interval following infestation. Losses of 20.1%, 14.5%, 11.9%, 10.4%, and 6.4% in this same interval were induced by *Aspergillus candidus, Penicillium palitans, P. rugulosum, Mucor racemosus* and *Aspergillus amstelodami,* respectively. The losses at the 2-week interval with 6 of the fungi were approximately one-half of these while those with *Penicillium palitans, P. rugulosum,* and *M. racemosus* were lower.

In the first trial, where the analyses were made at weekly intervals instead of at 2-weekly intervals as in the second trial, similar losses of organic matter were obtained. These losses were approximately linear with all the fungi over the 4-week period following inoculation. As in the second trial, *Aspergillus flavus, Penicillium chrysogenum* I, *P. chrysogenum* II, and *Aspergillus niger* were similar in their high rate of organic matter decomposition (fig. 1), causing approximately 40% losses of organic matter in 4 weeks, even though the first two and the last two fungi were tested at different times. The other fungi, which were also tested at different times, comprised a group with organic matter decomposition at approximately one-fourth of this rate.

Fat acidity changes

The high fat acidities of around 200 units and as high as 380 units presented in table I and figure 1 for the two trials compared favorably with the high fat acidities found occurring in molded shelled corn under commercial storage (**10**). The highest value in this instance of 273 units per 100 grams of dry matter was greater than any reported by Zeleny (**14**) for sample grade corn, but approached that for badly heated or *Diplodia zeae* infected corn. In the present experiments, 200 and more units of fat acidity were produced by *Aspergillus flavus, A. niger, Penicillium chrysogenum* I and *P. chrysogenum* II, the fungi which caused the greatest and most rapid organic matter losses and by *Aspergillus amstelodami* and *Mucor racemosus,* the fungi which caused lower organic matter losses. The remaining 3 fungi,

Penicillium palitans, P. rugulosum, and *Aspergillus candidus* produced fat acidity values of between 100 and 150 units (table I and fig. 1). These values were low in comparison with those produced by the other fungi, but approached the fat acidities found in the poorest of sample grade corn (**14**).

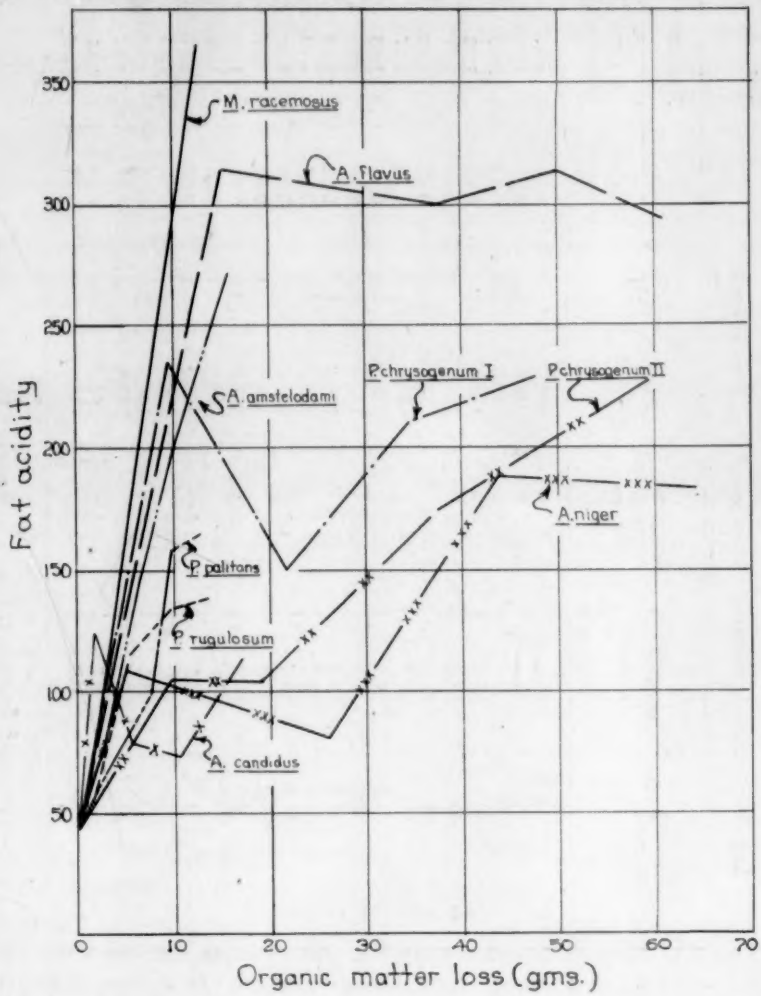

FIG. 1. Relation between organic matter loss and fat acidity at 4 successive weekly intervals. Data from the first trial.

As may be seen from the data in table I, the maximum fat acidity values produced by the different fungi were reached by approximately the end of the 2-week period, except for *Mucor racemosus,* which produced an even greater fat acidity by the end of the 4-week period. In the first trial, on the other hand, the maximum fat acidity values were reached within the first week by *Aspergillus flavus, A. candidus,* and *Penicillium chrysogenum*

TABLE I

ANALYSIS OF STEAM STERILIZED CORN MOLDED BY NINE FUNGI

FUNGUS	WEEKS OF DEVELOPMENT	ORGANIC MATTER LOSS		MOISTURE FORMED	MOISTURE CONTENT (WET BASIS)	FAT* ACIDITY	pH	NITROGEN*		
								WATER-SOLUBLE	WATER-INSOLUBLE	TOTAL
		gm.	*%*	*gm.*	*%*	*units*		*gm.*	*gm.*	*gm.*
None (control)	2	0†	0	0	32.7	43.9	6.0	0.17	1.96	2.13
	4	0†	0	0	32.8	43.3	5.9	0.16	2.00	2.16
A. flavus	2	36.2	26.4	24.6	47.5	187.9	5.7	0.48	1.39	1.88
	4	60.0	44.0	34.8	57.0	176.9	5.2	0.41	1.21	1.62
A. candidus	2	17.6	12.8	11.4	39.5	81.3	6.1	0.41	1.60	2.01
	4	27.5	20.1	12.8	42.2	74.8	5.8	0.39	1.57	1.96
A. amstelodami	2	5.0	3.6	4.8	35.1	384.1	5.9	0.15	1.86	2.01
	4	8.7	6.4	3.5	35.4	247.9	5.8	0.18	1.82	1.98
A. niger	2	28.8	21.0	23.4	45.4	180.5	3.4	0.53	1.40	1.93
	4	52.7	38.6	32.7	54.2	129.0	4.1	0.33	1.45	1.78
P. chrysogenum I	2	26.3	19.1	20.6	44.0	181.4	4.7	0.39	1.63	2.02
	4	53.1	38.9	31.2	54.0	136.4	5.1	0.40	1.47	1.87
P. chrysogenum II	2	27.3	19.8	19.7	44.0	252.8	4.9	0.74	1.34	2.08
	4	56.7	41.6	30.9	55.0	209.6	5.1	0.41	1.38	1.79
P. palitans	2	5.5	4.0	5.1	35.3	113.0	5.1	0.20	1.87	2.07
	4	19.7	14.5	10.8	39.9	120.6	5.4	0.33	1.75	2.08
P. rugulosum	2	5.2	3.8	3.1	34.6	104.9	5.5	0.26	1.87	2.13
	4	16.2	11.9	7.4	38.1	99.4	5.2	0.31	1.82	2.13
Mucor racemosus	2	2.2	1.6	2.7	34.0	286.2	5.8	0.30	1.81	2.11
	4	14.1	10.4	8.6	38.1	312.6	5.1	0.37	1.80	2.17

* On the individual culture basis.

† Dry matter content of individual control flasks at 2 weeks = 137.2 gm.; at 4 weeks = 136.5 gm.

I, within the third week by *P. rugulosum, P. palitans* and *Aspergillus niger* and beyond the fourth week by *A. amstelodami, Mucor racemosus* and possibly *P. chrysogenum* II (fig. 1). In both trials, no over-all relationship was evident between the quantity of organic matter lost and the fat acidities produced. The fungi continued to decompose organic matter even after the maximum fat acidities were produced (table I and fig. 1).

pH CHANGES

The pH changes of the molding corn were in the acid direction (table I). The greatest drop in pH (from the initial 6.0 to 3.4) was induced by *Aspergillus niger*. No relationship was evident with the different fungi between the changes in pH, the fat acidities and the quantity of organic matter decomposed.

NITROGEN CHANGES

The changes in the nitrogen content of the corn (table I) reflected the metabolic activities of the fungi. In the two trials, the water-insoluble nitrogen decreased while the water-soluble nitrogen increased. Total nitrogen decreased, presumably because ammonia was formed and escaped as a gas. Only 8% of the total nitrogen in the corn was initially water-soluble, while the remainder was water-insoluble.

After 2 weeks of fungus growth (table I), the water-soluble nitrogen proportion in the moldy corn was changed to 25.5%, 20.4%, 27.5%, 19.3%, and 35.6% by *Aspergillus flavus, A. candidus, A. niger, Penicillium chrysogenum* I and *P. chrysogenum* II, respectively, and to 9.7%, 12.2%, and 14.2% by *P. palitans, P. rugulosum* and *Mucor racemosus*, respectively. The first 5 fungi caused the greatest quantity of organic matter loss in this interval; namely, 26.4%, 12.8%, 21.0%, 19.1%, and 19.8% respectively; while the last 3 fungi were very weak in this capacity, causing 4.0%, 1.8%, and 1.6% losses, respectively. *Aspergillus amstelodami* caused a 3.6% loss of organic matter without increasing the proportion of water-soluble nitrogen in the moldy corn over the original. However, since there was a lowering of water-insoluble nitrogen in the corn, this indicated that some nitrogen was being mobilized by this fungus.

After 4 weeks of fungus growth the proportion of water-soluble nitrogen in the moldy corn was further increased only by those fungi which caused but slight organic matter losses at the 2-week period. With those fungi that were the greatest decomposers of organic matter the water-soluble nitrogen did not undergo any further increase but even decreased in amount. Autolytic processes (**2, 3, 6**) presumably were responsible for this latter result.

RELATION BETWEEN NITROGEN CHANGES AND ORGANIC MATTER LOSSES

HECK (**4**) has pointed out that 40% to 70% of fungus nitrogen is water-soluble. In view of this finding, and those just presented, the extent of the decreases in the water-insoluble nitrogen and the increases in the water-soluble nitrogen obtained in the molding corn might be considered

as indicators of the relative extent of fungus development. In figure 2 is presented the linear relationship between the quantitative loss of organic matter by the different fungi after 2 and 4 weeks' development and the decrease in the water-insoluble nitrogen. Linearity was also obtained, as shown in figure 3, between loss of organic matter and the increase in the amount of water-soluble nitrogen at the 2-week period. At the 4-week period, this linearity was not obtained presumably because of autolytic

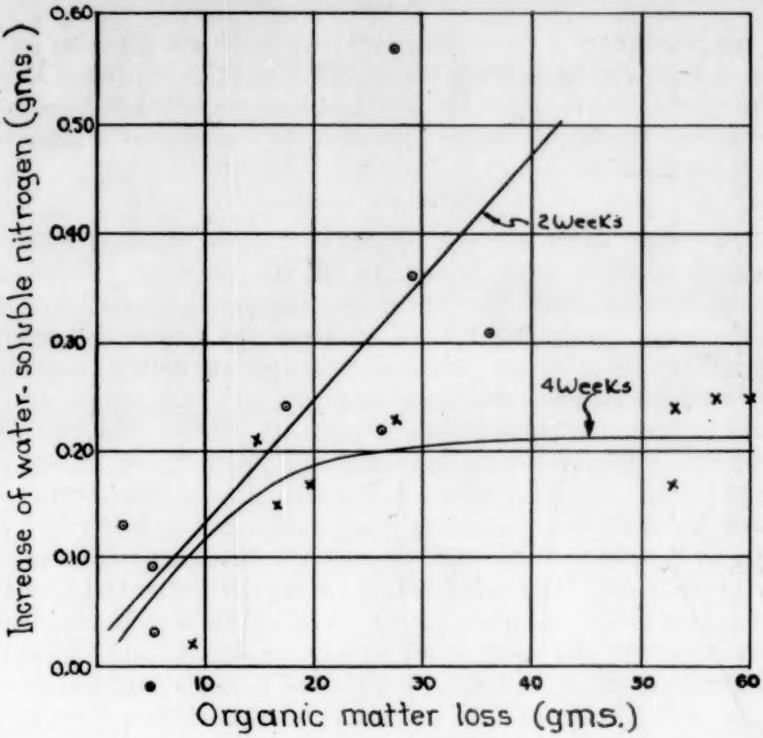

FIG. 2. Regression of increase in water-soluble nitrogen on organic matter loss after 2 and 4 weeks of fungus development. (Data from table I.)

processes surrounding the activities of *Aspergillus flavus, A. candidus, A. niger, Penicillium chrysogenum* I and *P. chrysogenum* II. Autolysis might be expected at this later period with these fungi because they were the most rapid growers and decomposers of organic matter. Continuation of growth activity eventually would result in the hyphae being in an unfavorable environment of depleted carbohydrate supply; in consequence they would undergo autolysis. This process in molds is known to result in nitrogen losses from the hyphae in the form of ammonia (**2, 3, 6**).

WATER FORMATION

The quantity of water that each fungus produced during its activity on corn is presented in table I. No special effort was made during this experi-

ment to prevent the loss of water by evaporation from each culture other than to plug the opening of each flask with a tight wad of cotton. Since the moisture content of the uninfested control corn remained unchanged after 2 and 4 weeks' standing in the laboratory, this suggested little or no water loss by evaporation.

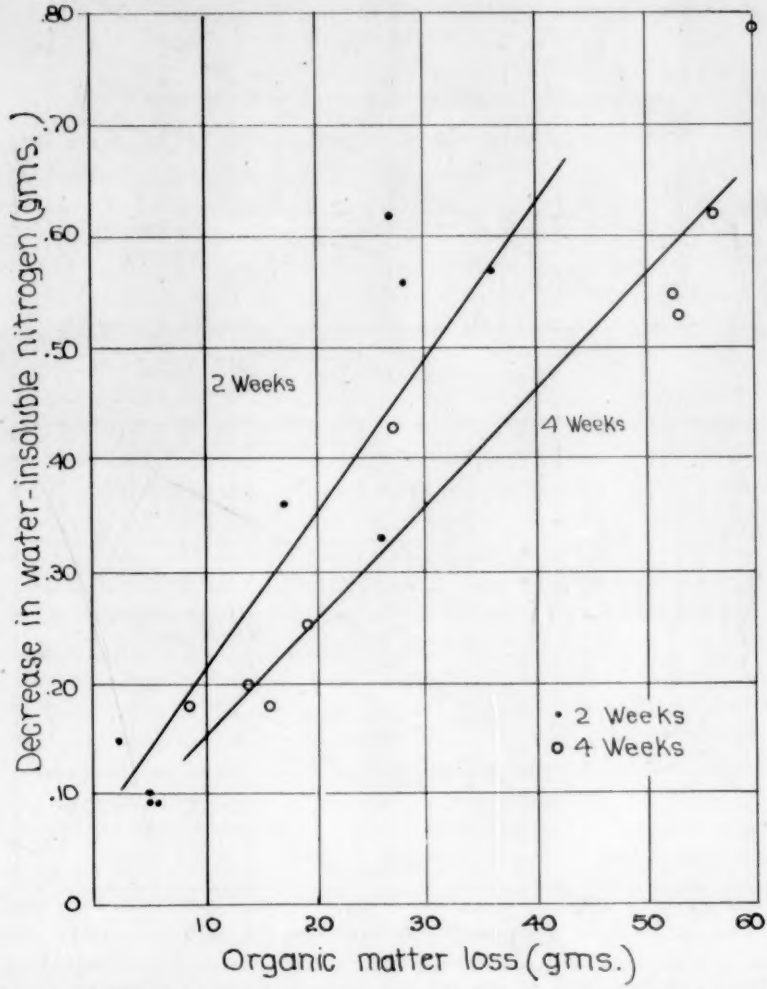

FIG. 3. Regression of decrease in water-insoluble nitrogen on organic matter loss after 2 and 4 weeks of fungus development. (Data from table I.)

The striking linear relationship between the percentage loss of organic matter induced by the different fungi after 2 and 4 weeks of development and the percentage of moisture (wet basis) in the molding corn at these two periods is presented in figure 4A. For every 1% loss of organic matter there was a corresponding rise of 0.55% moisture in the corn. The quantitative relationship between these two variables after 2 and 4 weeks of fungus

development and during the interval between the second and fourth weeks of development is shown in figure 4B. The ratio between the organic matter lost and the water formed was 1 : 0.701 at the 2-week period, 1 : 0.601 at the 4-week period, and 1 : 0.380 during the interval between the second- and

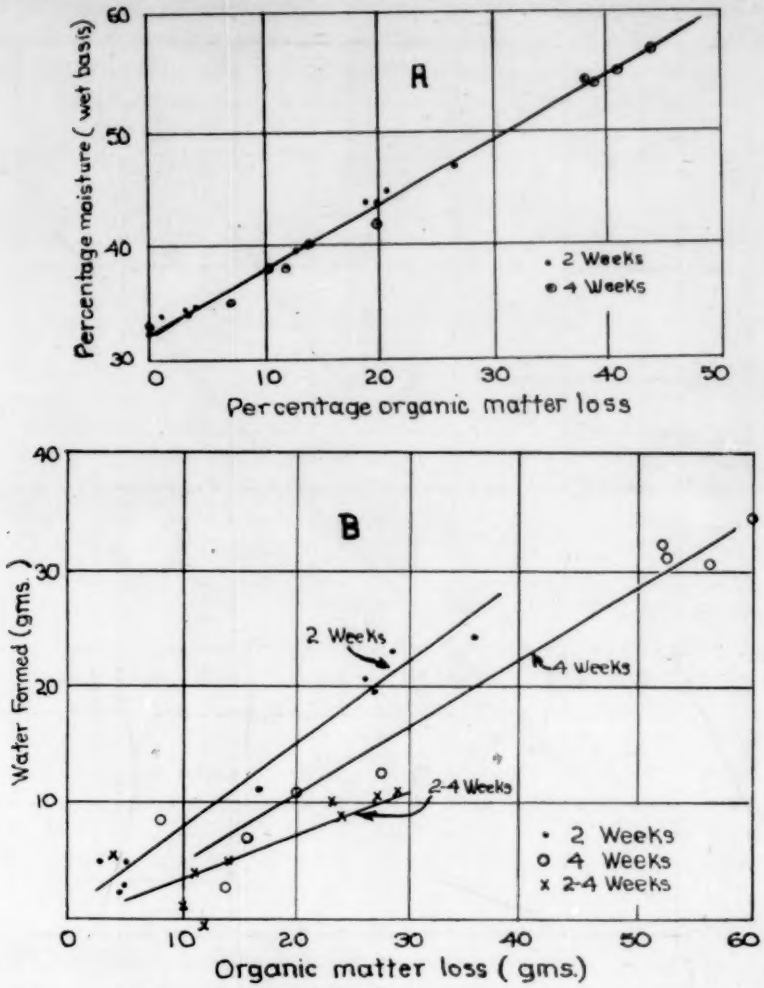

FIG. 4. A. Regression of percentage moisture (wet basis) on percentage loss of organic matter of corn digested by different fungi after 2 and 4 weeks. $Y = 0.55X + 32.63$. B. Regression of grams water formed by different fungi on grams organic matter loss after 2 weeks ($Y = 0.701X + 0.89$), 4 weeks ($Y = 0.601X - 1.546$) and during the 2 and 4 weeks' interval ($Y = 0.380X - 0.340$).

fourth-week periods. One gram of starch on complete oxidation should yield, theoretically, 0.555 grams of water while fat should yield water at better than a 1 : 1 ratio. The ratio of 1 : 0.701 obtained at the 2-week period suggests utilization of both starch and fat by the fungi. At the beginning

of this study molds were observed to be developing in greatest abundance at the germ position on the kernels where the fats and oils are concentrated. The ratio of 1:0380 obtained during the interval between the second- and fourth-week periods would indicate the incomplete oxidation of organic matter if there was negligible loss of water through evaporation from the culture flask as suggested by the noninfested controls. The ratio of 1:0.601 obtained at the 4-week period would represent the average result of all these processes.

Growth of the fungi on Czapek-Dox medium

The results of the experiments on corn reported above revealed considerable variation among the fungi in the quantity of organic matter decomposed and the fat acidity produced. To supplement these observations these same fungi were cultured on Czapek-Dox solution to determine whether or not their carbohydrate-utilizing and acid-producing powers on this medium would be similar to that on corn. The results for all the fungi except *Mucor racemosus* and *Aspergillus amstelodami*, which failed to develop in Czapek-Dox medium, are presented in table II.

In general, except for the differences in the nature of the two media and the different analytical methods used, the responses of the individual fungi were similar on the two media. *Aspergillus niger*, *Penicillium chrysogenum* I, and *P. chrysogenum* II produced a rapid lowering of the pH and a high titrable acidity on Czapek-Dox medium and on corn. *Aspergillus niger* gave essentially similar results on the two media except that comparatively low fat acidities were produced on corn. This latter result was probably because such benzene-insoluble acids as oxalic, citric, and gluconic (which are notable products of *A. niger* metabolism) were excluded from the determination. *Penicillium palitans* and *P. rugulosum* produced moderate titrable acidities on Czapek-Dox medium and on corn. The initial pH rise on Czapek-Dox medium was presumably due to the initially small acid-production and the liberation of the sodium ions from $NaNO_3$ by these fungi. Acidic substances were later produced, however, because the pH was lowered on prolonged growth of the fungus. These results were in general agreement with those on corn. *Aspergillus flavus* produced considerable quantities of kojic acid, which is a weak acid, on Czapek-Dox medium and on corn. The quantitative production of this acid (**1**) on Czapek-Dox medium may be deduced from table II by taking the difference between the "glucose" values determined by the copper and the iodometric methods (**1**). *Aspergillus candidus* produced negligible amounts of titrable acidity in Czapek-Dox medium and as a result the pH of the medium was thrown over to the alkaline side. Moderate fat acidities, however, were produced on corn, presumably because this fungus acted on the fat constituents of the germ.

The glucose utilization rates in Czapek-Dox medium were initially most rapid with *Penicillium chrysogenum* I, *P. chrysogenum* II, *Aspergillus niger*, and *A. flavus*, the 4 fungi most active in decomposing corn organic matter.

TABLE II

ANALYSES OF CULTURES OF 7 FUNGI DEVELOPING ON CZAPEK-DOX MEDIUM

	Weight of mycelium			pH of medium*			Glucose remaining†						Titrable acidity‡
							Copper method			Iodometric method			
	1 wk.	2 wk.	4 wk.	1 wk.	2 wk.	4 wk.	1 wk.	2 wk.	4 wk.	1 wk.	2 wk.	4 wk.	4 wk.
	mgm.	*mgm.*	*mgm.*				*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
Penicillium palitans	94.4	124.5	149.0	6.09	5.28	4.25	871	638	235	867	624	277	6.40
P. rugulosum	94.2	152.4	189.3	5.55	4.39	3.86	870	542	105	854	528	129	6.16
P. chrysogenum I	69.6	132.0	176.3	3.66	3.33	3.84	650	121	6	660	153	53	10.00
P. chrysogenum II	99.7	173.4	190.2	3.62	3.30	3.68	504	42	6	555	54	35	8.92
Aspergillus flavus	145.7	144.0	138.7	5.70	5.21	5.71	712	630	464	705	832	876	7.68
A. niger	169.2	176.0	190.3	3.12	2.86	3.07	587	570	399	620	562	410	20.20
A. candidus	40.2	56.2	119.2	7.36	7.32	6.80	1026	922	628	1050	969	676	1.20

* Determined on the culture filtrate diluted to 100 cc. Initial pH, value 4.26.
† Initial glucose supply was 1164 mgm.
‡ Milliliters of 0.1 N NaOH to phenolphthalein per culture.

While these initial rates on Czapek-Dox medium were continued with the two cultures of *Penicillium chrysogenum* to the near complete utilization of sugar, the rates with *Aspergillus niger* and *A. flavus* were reduced markedly at the point of approximately 50% sugar utilization. Presumably, unfavorable changes occurred within the liquid medium supporting these latter two fungi so that their further activity was hindered. *Penicillium palitans, P. rugulosum,* and *Aspergillus candidus,* on the other hand, utilized sugar at a constant, slower rate over the 4-week culture period. These slower sugar utilization rates conformed to the slower rates of corn organic matter decomposition by these same fungi.

Discussion

The loss in organic matter and the changes in the moisture content and fat acidity of molding corn under pure culture conditions compared favorably with similar changes noted in molded corn found in storage bins under natural conditions (**10**). The high moistures and fat acidities observed in the molded corn in the bins (**10**) may be attributed in large part to the metabolic activities of the organisms themselves. The higher water-soluble nitrogen content of the molded corn observed in the laboratory reflected the activities of these organisms, and this would suggest that the higher amino acid content found in sample grade corn (corn having over 15% of damaged kernels) as against No. 1 to No. 5 yellow corn as noted by ZELENY and COLEMAN (**14**) may also be the product of fungus activity.

The effect of mold growth on the corn oil was not determined in the present study. WINTON, BURNET, and BORNMANN (**12**), RABAK (**9**), and WILLITS and KOKOSKI (**11**) have reported the quantitative reduction of the oil content in corn. MCHARGUE (**8**) noted that the oil extracted from moldy cornmeal possessed a musty odor, was dark brown in color, and titrated 56.8 ml. alkali per kilogram of oil. Results similar to these were obtained for shelled corn by RABAK (**9**). A chemical analysis of the oil extracted from spoiled corn by that investigator revealed increases in specific gravity, refractive index, free acids, soluble acids, hydroxylated acids, and unsaponifiable constituents; decreases in volatile acids, insoluble acids and unsaturated acids were noted. WILLITS and KOKOSKI (**11**) observed higher saponification values and lower iodine numbers of the oil in spoiled cornmeal.

The relationship between the loss of organic matter and the formation of water in the molded corn indicated, as expected, that water was being liberated during mold development. This might suggest that molds would be able to create favorable moisture conditions for their own development even in initially fairly dry corn, providing that enough moisture was available in the kernels to allow them to initiate fungal development.

The changes in the fat acidity of molded corn reflected, in large measure, the acid-producing capabilities of the individual molds themselves. While the term "fat-acidity" implies that the origin of these fatty acid constituents resides in the degradation of the fats (**7**), its determination remains

quite empirical as it includes all the benzene-soluble acidic substances regardless of their origin. No distinction is made as to the extent to which these constituents arise directly from the fat of the corn itself and from the carbohydrates as by-products of mold metabolism. The recognized agents responsible for the formation of fatty acids from the fat have been (a) the enzymes normally present in the food product studied, (b) the direct participation of atmosphere oxygen, and (c) the fat-hydrolyzing lipases liberated by the microorganisms present. The present work suggests that perhaps part of the fat-solvent soluble acids produced in molding corn might include the acids produced by the microorganisms incident to their metabolism of corn carbohydrates.

Summary

Pure cultures of 9 fungi isolated from naturally molded corn were grown in the laboratory for a 4-week period on steam-sterilized corn initially adjusted to approximately 32 percentage moisture content. Analyses were made at weekly and bi-weekly intervals for changes in the amounts of water, organic matter, fat acidity, pH, water-soluble, and -insoluble nitrogen. Seven of these fungi were also grown on Czapek-Dox liquid medium to compare their carbohydrate-utilizing and acid-producing powers on this medium with the changes they induced in corn.

Penicillium chrysogenum I, *P. chrysogenum* II, *Aspergillus niger*, and *A. flavus* were most active in decomposing corn organic matter, producing losses of 40% to 45% within a 4-week period. These same fungi on Czapek-Dox medium were initially the most rapid utilizers of glucose.

A positive linear relationship was found between the percentage loss in organic matter and the percentage of water (wet basis) in the moldy corn. A 1% decrease in organic matter resulted in a 0.55% increase of water (wet basis). The relationship between the quantity of organic matter lost and quantity of water formed proved linear over 2 and 4 weeks of fungus development. The ratios between these two variables were 1 : 0.701 at the 2-week period and 1 : 0.601 at the 4-week period.

Fat acidities in the corn were increased by all the fungi tested in this series of experiments. The greatest fat acidities were produced by the 4 fungi that decomposed organic matter the most rapidly, and by *Mucor racemosus* and *Aspergillus amstelodami* which decomposed the organic matter slowly.

Linear relationships were noted at the early period of fungus development between the losses of organic matter and the changes in the amounts of water-soluble and -insoluble nitrogen. Nonlinearity between organic matter loss and water-soluble nitrogen was noted at the later period.

General agreement between the pH and fat acidity of molded corn and the pH and titrable acidity of Czapek-Dox medium was obtained with the different fungi.

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CHANGES IN THE CHLOROPHYLL AND CAROTENE CONTENTS OF CURING BURLEY TOBACCO CUT AT DIFFERENT STAGES OF MATURITY[1]

R. N. JEFFREY AND R. B. GRIFFITH

(WITH ONE FIGURE)

Received June 6, 1945

Introduction

Changes in the chemical composition of burley tobacco which take place in curing are being investigated at the Kentucky Agricultural Experiment Station in an attempt to understand the physiology of curing and its effect on leaf quality. Color is recognized as one of the most important factors in the determination of leaf quality, any retention of green pigment in the cured leaf greatly reducing its value. The chlorophylls are the green pigments in normal tobacco, and the investigations reported in this paper were made primarily to determine the rate and amount of change in these pigments during air curing on the stalk of tobacco cut at three stages of maturity. The development of a method for determining carotene from the same solution used in the chlorophyll determinations made possible the study of this constituent.

This study of the catabolic changes of plant pigments is of general biochemical and physiological interest. Numerous articles have appeared reporting the rates of formation of these pigments under different conditions, but studies on pigment disappearance are rare. In 1918 WILLSTÄTTER and STOLL (**5**) reported that the chlorophyll content of yellowed leaves was less than of green leaves. They did not, however, follow closely the loss of either chlorophyll or carotenoids. GUTHRIE (**2**) investigated the pigment changes in potted plants when placed in the dark as compared with others remaining in the light. His results indicated that tomato plants lost about 25% of their chlorophyll in four days, soybean plants about 70% in eight days, and yellow coleus practically none in eight days.

Methods

HARVESTING AND CURING TECHNIQUE

Kentucky No. 16 burley tobacco was cut at three different stages of maturity as determined by experienced growers: (1) immature by about 10 days, (2) mature, and (3) overmature by 10 days. These lots of tobacco were topped to approximately 20 leaves, and to a height of about 136 cm. (4.5 ft.) two, 14, and 24 days respectively before cutting. The entire plant was cut just above the ground and the stalk "speared" onto a stick and hung to cure with the plant in an inverted position. Spearing

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

caused a split in the stalk about a foot long. Although the majority of the plant cells remained alive for some time, the more mature basal leaves, which had already started to turn yellow before cutting, died first, beginning at the tips. The yellowing and subsequent death of the cells then progressed gradually to the more immature leaves. The axillary buds near the tip of the stalk were still alive at the end of the curing period. Thus the curing process for this type of tobacco is one of gradual starvation and desiccation, accompanied by extensive chemical changes which may be catalyzed by the enzymes of the living plant.

The tobacco, after wilting in the field for 24 hours following cutting, was cured in the air-conditioned curing chambers described by O'BANNON (**4**) at 68% relative humidity and a temperature of 23.8° C. (74.9° F.). JEFFREY (**3**) found these conditions to be within the optimum range for the production of highest commercial quality cured leaf.

SAMPLING AND ANALYSIS

To sample two different stages of leaf maturity on each plant analytical samples were taken from two different levels. One, called the "basal leaf sample," was from each plant's basal three leaves which were sound and therefore likely to remain on the plant during curing; the other, designated as the "top leaf sample," was from the 5th, 6th, and 7th leaves from the point of topping. After curing, leaves from these positions on the stalk were graded chiefly as "trash" and "bright leaf," respectively. Leaves to be used as samples were measured and tagged in the field a day before cutting. Duplicate samples were taken in the field at cutting time, five days before the overmature plants were cut, and in the curing chambers. The frequency of sampling in the chambers was dependent on the rate of change observed. A sample consisted of four leaves, each taken from a different plant. The midribs were removed. The right halves of two of the leaves and the left halves of the other two were used for pigment extraction, and the remaining halves were placed in an oven at 65° C. for 48 hours to determine the percentage dry weight.

The leaf material was analyzed for chlorophylls *a* and *b* and for carotene using the method described by GRIFFITH and JEFFREY (**1**). By this method the pigments are extracted from the sample with acetone in a Waring blender and transferred to ether. Chlorophyll is determined in a dilution of the ether solution by means of spectrophotometric readings at the red absorption maxima of chlorophylls *a* and *b*, and carotene is determined spectrophotometrically after separation from the other pigments in the ether solution by the use of a chromatographic adsorption column. The estimation of xanthophyll had not been included in the method at the time this study was conducted.

EXPRESSION OF DATA

Since burley tobacco is cured on the stalk, the green or dry weight of the samples to be used for analysis could not be determined at cutting time.

YOUNG and JEFFREY (6) have shown that tobacco leaves cured under the same conditions used in this experiment lose 30% of their dry weight during the curing process; consequently, the oven dry weight does not constitute a satisfactory basis for reporting results. They found, however, that there was a high degree of correlation between the product of length and width of leaf and leaf area, and that the leaf area was a satisfactory basis for reporting changes that occur in curing tobacco. The leaf areas were calculated, using the equation:[2]

$$y = \frac{0.65\,x}{10{,}000} + 0.006$$

where y is the leaf area in square meters and x is the product of the length and width of the leaf expressed in centimeters.

Results

The chlorophyll and carotene contents of the top leaves at various times during the curing of plants cut when immature, mature and overmature are presented in table I. Similar results for basal leaves are presented in table II. The results expressed on an area basis are shown graphically

TABLE I

CHLOROPHYLL AND CAROTENE CONTENT OF TOP LEAVES AT VARIOUS STAGES OF CURING ON THE STALK FOR TOBACCO PLANTS CUT AT THREE STAGES OF MATURITY. RESULTS CALCULATED ON THE BASIS OF OVEN DRY WEIGHT AND OF FRESH LEAF AREA. LEAVES IN CURING CHAMBER EXCEPT AS NOTED

AGE	DAYS FROM FIRST CUTTING	OVEN DRY WEIGHT	WEIGHT BASIS		CHLOROPHYLL *a*	AREA BASIS	
			CHLOROPHYLL	CAROTENE		CHLOROPHYLL	CAROTENE
		%	*mg./gm.*	*mg./gm.*	%	*mg./m.*^{2}	*mg./m.*^{2}
	0.0*	16.2*	8.28 *	0.464*	72.0*	277.0*	15.4*
	2.0	16.1	7.18	0.444	70.1	227.0	14.0
	5.1	16.5	6.26	0.393	66.0	196.0	12.3
Immature	8.5	17.5	5.16	0.354	69.0	156.0	10.7
	15.1	24.6	1.46	0.263	68.5	39.0	7.0
	17.9	35.9	0.27	0.174	58.6	6.0	4.8
	21.0	51.4	0.52	0.183	67.3	13.0	4.5
	39.0	89.8	0.22	0.139	58.1	5.0	3.3
	10.0*	17.5*	5.10 *	0.299*	71.0*	188.0*	11.0*
	12.0	16.7	4.19	0.298	71.9	126.0	9.0
Mature	15.1	17.8	2.45	0.190	64.3	78.0	6.1
	18.5	34.4	0.24	0.119	67.1	6.4	3.2
	28.3	86.7	0.022	0.075	62.2	0.6	2.0
	34.0	86.1	0.020	0.070	58.3	0.5	2.0
	10.0†	17.5†	5.10 †	0.299†	71.0†	188.0†	11.0†
	14.8†	15.6†	4.46 †	0.265†	72.6†	160.0†	9.5†
	20.0*	16.6*	2.76 *	0.200*	72.6*	92.0*	6.7*
Overmature	21.9	18.5	1.88	0.156	71.6	62.0	5.2
	24.5	30.2	0.21	0.089	64.2	6.3	2.7
	27.9	79.1	0.027	0.079	61.4	0.8	2.3
	39.0	89.3	0.014	0.064	57.4	0.4	1.9

* Cut.
† In field.

[2] An error was present in the previously published form of this equation (6).

in figure 1. The left part of this figure shows the changes which take place in the chlorophyll content of the top leaves. In the first 17.9 days after cutting, the chlorophyll content of the immature tobacco decreased at an average rate of 15.1 mg./m.^{2} per day. Chlorophyll was lost by the mature tobacco at the rate of 21.4 mg./m.^{2} per day in the first 8.5 days of curing, and by the overmature tobacco at the rate of 16.9 mg./m.^{2} per day in the first 5.1 days of curing. The rate of chlorophyll loss in the tobacco growing in the field was 8.9 mg./m.^{2} per day in the first 10 days and 9.6 mg./m.^{2} per day in the second 10 days. The tobacco cut when immature and that

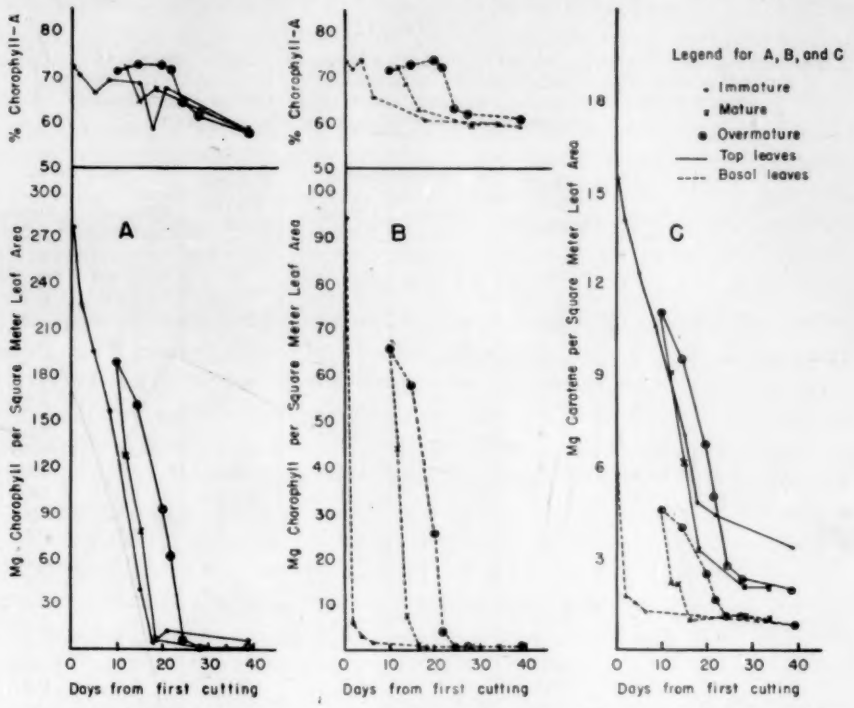

FIG. 1. Chlorophyll changes in top leaves (A), in basal leaves (B), and carotene changes in top and basal leaves (C) of curing burley tobacco cut when classified by experienced growers as immature, mature (10 days later), and overmature (20 days later).

cut when mature reached essentially the same chlorophyll content at the same time, even though the mature tobacco was cut 10 days later. Less time was required for chlorophyll destruction in the mature and overmature tobacco than in the immature tobacco, curing was more uniform, and the quality of leaf was better. The top leaves of the tobacco cut while immature still contained chlorophyll at the end of the curing period, incipient house-burn had occurred, and even the quality of the basal leaves was low.

The changes in the percentage of chlorophyll *a* based on total chlorophyll, are shown in the upper part of figure 1. The point representing the immature tobacco after 21 days is probably in error, since in the other five

instances in this and in the next graph representing basal leaves, the percentage chlorophyll *a* fell from about 70% to below 63% at about the same time that the total chlorophyll concentration ceased to fall. The oven dry weight in the leaf web was about 30% to 35% in all three cuttings of tobacco when the total chlorophyll content and the percentage chlorophyll *a* approached minimum values.

Changes in the chlorophyll content of the basal leaves are presented in the central part of figure 1. The initial chlorophyll content of these older leaves was much lower than that of the top leaves, and the time required

TABLE II

CHLOROPHYLL AND CAROTENE CONTENT OF BASAL LEAVES AT VARIOUS STAGES OF CURING ON THE STALK FOR TOBACCO PLANTS CUT AT THREE STAGES OF MATURITY. RESULTS CALCULATED ON THE BASIS OF OVEN DRY WEIGHT AND OF FRESH LEAF AREA. LEAVES IN CURING CHAMBER EXCEPT AS NOTED

Age	Days from first cutting	Oven dry weight	Weight basis		Chlorophyll *a*	Area basis	
			Chlorophyll	Carotene		Chlorophyll	Carotene
		%	*mg./gm.*	*mg./gm.*	%	*mg./m.²*	*mg./m.²*
Immature	0.0*	11.3*	3.29*	0.211*	73.0*	94.2*	6.05*
	2.0	13.5	0.23	0.069	71.7	5.9	1.73
	3.9	31.0	0.11	0.051	73.1	3.3	1.57
	6.4	47.1	0.06	0.042	65.4	1.5	1.20
	18.1	86.9	0.03	0.043	60.1	0.8	1.02
	39.0	89.8	0.03	0.030	59.0	0.7	0.73
Mature	10.0*	12.3*	2.26*	0.158*	71.2*	65.5*	4.57*
	12.0	13.7	1.77	0.087	71.3	43.6	2.16
	13.9	21.2	0.30	0.085	67.9	7.4	2.07
	16.4	79.8	0.06	0.042	62.8	1.3	0.91
	28.3	87.4	0.03	0.043	59.0	0.8	1.06
	34.0	88.0	0.03	0.043	59.9	0.7	0.96
Overmature	10.0†	12.3†	2.26†	0.158†	71.2†	65.5†	4.57†
	14.8†	11.8†	1.71†	0.121†	72.5†	57.5†	4.02†
	20.0*	11.9*	0.92*	0.090*	73.6*	25.4*	2.47*
	21.9	20.7	0.16	0.063	72.0	4.0	1.58
	24.5	79.9	0.04	0.041	62.9	0.8	1.00
	27.9	86.0	0.04	0.043	61.5	1.0	1.06
	39.0	90.5	0.02	0.027	60.5	0.4	0.67

* Cut.
† In field.

for its loss was less than in the top leaves. In the first two days after cutting, the rate of chlorophyll loss in the basal leaves of the plants cut while immature was 44.3 mg./m.² per day. In the first 3.9 days of curing the rate of loss in the basal leaves of the mature tobacco was 14.9 mg./m.² per day. While the overmature tobacco was still in the field the rate of loss was 4.0 mg./m.² per day and in the first 1.9 days after cutting the rate of loss was 11.3 mg./m.² per day. Except for the immature tobacco the rate of loss per day in the basal leaves was less than the rate of loss in the top leaves, but because of lower initial chlorophyll values the length of time necessary for chlorophyll decomposition was less. The extremely rapid rate of 44.3 mg./m.² per day of chlorophyll loss in the immature basal leaves was over

twice as great as any other rate of loss. Translocation of some substance or substances to the immature top leaves is the only explanation that can be offered for this rapid rate. The smaller rate of loss in the basal leaves of the plants cut when mature or overmature was probably due to the greater maturity and lower initial chlorophyll content of these plants. After five days of curing in plants of all stages of maturity there was very little change in chlorophyll content of the basal leaves.

The changes in percentage of total chlorophyll of the basal leaves which is chlorophyll *a* are shown in the upper part of figure 1. The sharp drop in percentage chlorophyll *a* in these basal leaves corresponded much more definitely than in the top leaves to the time at which the total chlorophyll reached a low value. It may be seen that the dry matter at this time is about 30% to 35% (table II), as it was also in the top leaf samples. However, decrease in chlorophyll values does not cease altogether at this point. In these experiments the chlorophyll concentrations reached a low value some time before the leaves became dry. Other experiments in the curing chambers have indicated that this would not have been true if the relative humidity of the air surrounding the curing plants had been low instead of optimum.

The results of the carotene determinations are presented in figure 1, C. The upper group of three curves represents the changes in the top leaves, while the lower group of three curves represents the changes in the basal leaves. Each set of curves is similar to the corresponding set of chlorophyll curves; however, the carotene does not appear to be so rapidly nor so completely destroyed as is the chlorophyll. Thus the yellowing of tobacco in the field and in curing is due in part to a more rapid and complete disappearance of chlorophyll than of carotene. At the time the break in the chlorophyll concentration curve occurs, the carotene content has been reduced to about one quarter its former value. A break occurs in the carotene concentration curves at this same time, corresponding to 30% to 35% dry matter, but these curves show a greater downward trend after this point has been reached than do the chlorophyll curves.

Discussion

The decrease in the calculated chlorophyll *a* percentage shown in the upper part of figure 1 may not appear to be very large, but there is reason to believe that the actual decrease is greater than is shown by these figures. All chlorophyll concentrations and percentage chlorophyll *a* values were calculated by means of equations based on the spectrophotometric absorption values at the red maxima of pure chlorophylls *a* and *b*. In green tobacco leaves no pheophytins were found, so satisfactory values were obtained by means of these equations. A chromatographic study of the pigments present in cured tobacco showed evidence of more pheophytin *a* than of chlorophyll *a*, though no pheophytin *b* was found. The absorption of pheophytin *a* at the red maximum of chlorophyll *a* is about half the latter, which would result in a calculated chlorophyll concentration by the method used, larger

than the true concentration of chlorophyll *a* plus *b*, but smaller than the concentration of the chlorophyll plus pheophytin *a*. This was confirmed by the concentration values calculated from the absorption values at 600 mμ, where the absorption of pheophytin *a* was more nearly equal to that of the chlorophylls. Close agreement was obtained between total chlorophyll concentration calculated from the readings at 600 mμ and at the *a* and *b* maxima on fresh leaf, but on the cured tobacco samples the concentration values determined at 600 mμ were larger than those calculated by the equations based on the chlorophyll maxima. Similarly, the percentage of chlorophyll *a* calculated by means of the equation is larger than the percentage of the chlorophyll which is really chlorophyll *a;* consequently the change in percentage chlorophyll *a* is really greater than that shown in the tables.

The break in the carotene curves is just as definite as that in the chlorophyll curves but the cause is more difficult to explain. At the time the break occurred the carotene values were about one-fourth as high as in the corresponding samples at cutting time. It does not seem probable that this concentration was low enough to affect the rate of the reaction sharply. Nor does it seem probable that the moisture content was low enough to cause such a marked reduction in reaction rate. The oven dry weight at this time was 30% to 35% and moisture was 65% to 70%. The moisture content threshold below which many other chemical reactions in cured tobacco leaf are inhibited, is approximately 80% dry matter and 20% moisture. It is possible that the carotene had undergone some change, but it was still sufficiently similar to true *beta* carotene to behave normally in the magnesium oxide-celite adsorption column. No light absorption studies were made of the carotene fraction from cured tobacco. Another possible explanation of the results is that the rate of carotene destruction may be connected in some way with the chlorophyll concentration, since the break in the two curves seems to occur at the same time. This could be a case of catalysis or it could be related to the existence of a definite chromoprotein molecule containing both chlorophyll and carotene, such as has been suggested by many workers.

Summary

1. A study was made of the changes in chlorophyll and carotene content of the leaves of stalk-cut burley tobacco during the air-curing process. Leaves from the upper and lower parts of plants, cut when immature, mature, and overmature, were analyzed. The initial chlorophyll content of the leaves decreased with increasing maturity, and was higher in the upper than in the corresponding basal leaves.

2. The rate of loss of chlorophyll in the top leaves was not greatly affected by maturity, and as a result, low chlorophyll concentrations were reached in a shorter time of curing in the overmature than in the immature plants.

3. The highest rates of chlorophyll loss were observed in the basal leaves of immature plants, indicating that translocation of some substances to the immature leaves may have occurred.

4. As the total chlorophyll content of the leaves reached low values, the percentage of chlorophyll *a* based on total chlorophyll decreased.

5. The shapes of the curves showing the carotene content of the leaves were very similar to the corresponding chlorophyll curves for leaves of each degree of maturity, though the carotene did not disappear so completely as the chlorophyll.

6. A change in rate of loss of carotene and of chlorophyll and in the proportion of chlorophyll *a* all occurred at a fairly definite moisture content, though the time required to reach this point depended upon the maturity of the leaves.

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INFLUENCE OF TEMPERATURE ON GROWTH AND ALKALOID CONTENT OF CINCHONA SEEDLINGS

HAROLD F. WINTERS, ARNAUD J. LOUSTALOT, AND NORMAN F. CHILDERS

(WITH FOUR FIGURES)

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Introduction

The importance of temperature as an environmental factor in the growth of Cinchona, the tree from which quinine is obtained, may be realized from a study of its natural environment. The genus is native to a region extending over 1,500 miles along the slopes of the Andes Mountains of South America. The various species occur at altitudes ranging from 2,500 to 9,000 feet above sea level (**4**). Relatively cool temperatures, high rainfall and high humidity prevail in these areas. In Java, where Cinchona has reached the peak of cultivation, the most suitable elevation is from 3,000 to 7,000 feet (**1**). Temperature conditions are relatively uniform from one month to the next. A mean temperature of 63° F. was the average over a period of 11 years at the Government Cinchona Station located at Tjinjiroean, Java, at 5,200 feet elevation (**2**).

Materials and methods

Three air-conditioned chambers 7 by 9 and 7 feet high, were constructed within a standard even-span greenhouse (fig. 1). The chambers were built

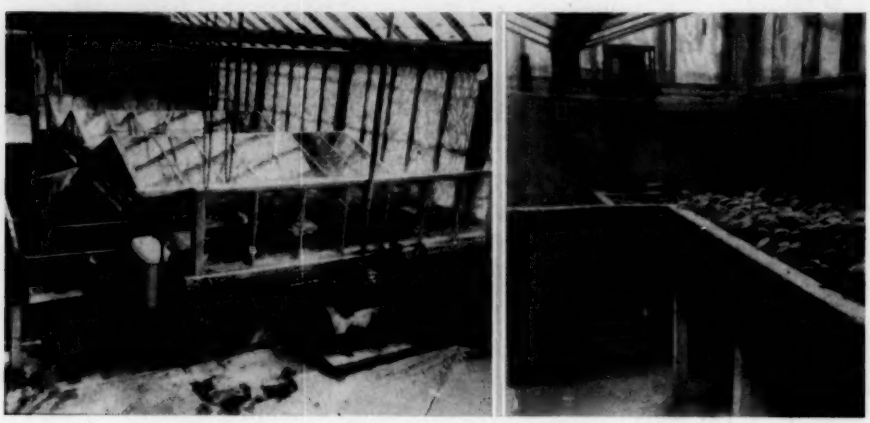

FIG. 1. (Left) Three air-conditioned chambers in which Cinchona seedlings were grown. (Right) Interior of one chamber showing temperature-control equipment and seedlings.

so that one-half their height was below the ground floor of the greenhouse. The tops and part of the sides of the chambers consisted of double glass with partitioned dead air spaces between.

The desired temperatures were uniformly maintained within each chamber by standard air-conditioning equipment. Fans on the diffusers operated continuously. When a temperature higher than the prevailing outside temperatures was desired in a chamber, small heating coils were turned on and the refrigeration system operated against this small flow of heat, thus main-

TABLE I

APPEARANCE OF *Cinchona ledgeriana* AND *C. pubescens* SEEDLINGS GROWN AT THREE TEMPERATURE LEVELS

ENVIRONMENT	TEMPERATURE				*C. ledgeriana*	*C. pubescens*
	SUMMER*		WINTER†			
	NIGHT	DAY	NIGHT	DAY		
	°F.	°F.	°F.	°F.		
Warm chamber	70	80	65	80	Many necrotic spots on older leaves during summer. No necrotic spots at end of experiment. Older leaves shed in summer. Thin leaves. Stems slender and weak. Poor root development.	Vigorous strong stems. Large leaves, tending to shade the weaker plants. Long internodes. Good root development.
Medium chamber	65	75	60	75	Occasional necrotic spots during summer, none in winter. Vigorous, strong growth. Good root development.	Sturdy healthy plants. Good root development.
Cold chamber	60	70	55	70	Stocky plants with thick stems and undulate leathery leaves. Leaves tinged with red. Excellent root development.	Stocky plants. All leaves retained. Short internodes. Excellent root development.
Greenhouse‡	.....	.....	.....	.....	Many necrotic areas on older leaves in summer. Plants shed all but 1 or 2 pairs of leaves. Poor root development.	

* Maintained from April through September.
† Maintained from October through March.
‡ No control.

taining a straight-line temperature level. During the first few months of the experiment it was necessary to use the coils for about three hours each morning in order to gradually raise air temperatures in the chambers to the desired summer-period levels.

Temperatures were thermostatically regulated for night and day and for summer and winter (table I). Thermostat settings were made manually at 8:00 A.M. and 4:00 P.M. daily. About 6 hours were usually required for the temperature in the chambers to reach the night levels, due to the simultane-

ous heavy drain on the compressor by all chambers. The day temperatures were reached in 2 to 4 hours depending upon the sky conditions.

Several times during the experiment leaks developed and the system was out of operation for short periods, during which time all plants were

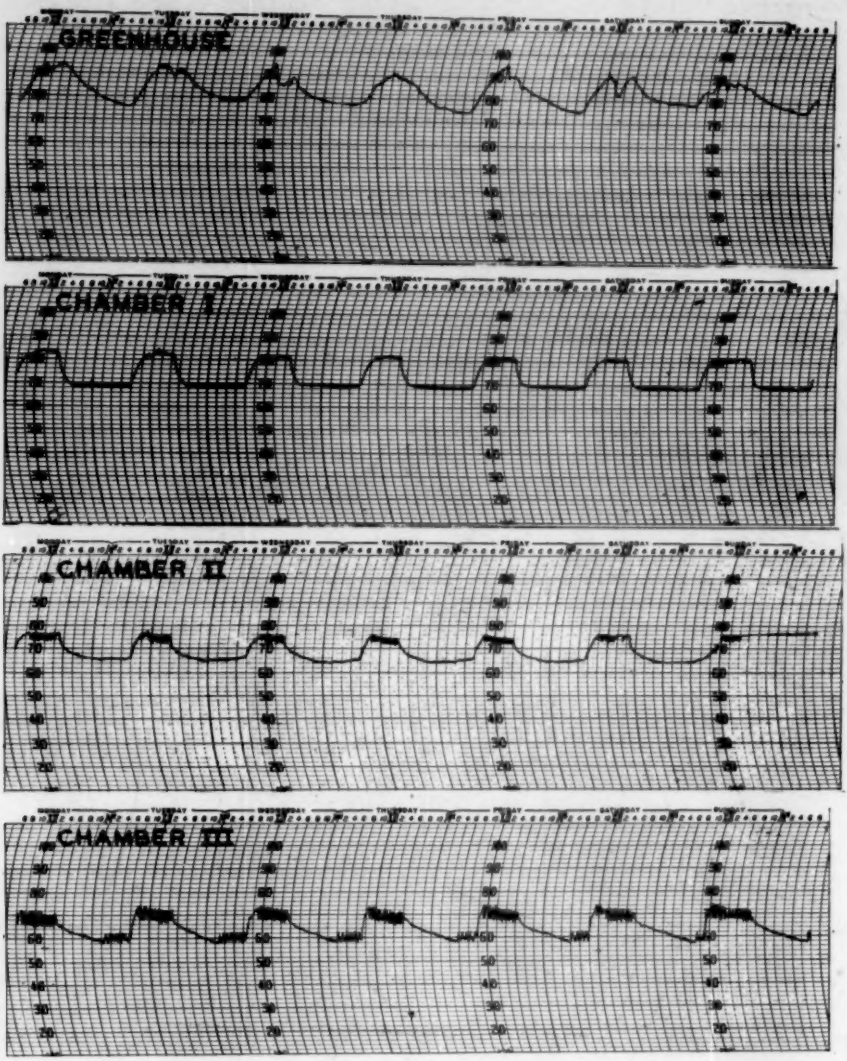

FIG. 2. A typical chart of temperatures in the greenhouse outside the chambers and sample temperature charts from warm, medium, and cold chambers. Data taken during summer period; in winter period night temperatures were 5° lower in chambers.

subjected to outside air-temperature conditions. As a whole, however, the experimental temperatures were maintained fairly closely. Continuous thermographic records were kept in each chamber and representative charts from the chambers and the outside greenhouse are given (fig. 2). Relative

humidity was maintained as near 100% as possible by spraying the floors with water.

Concrete benches within the chambers were filled with a soil composed of equal parts of loamy-clay soil, silica sand, peat moss, and leaf mold. Forty seedlings of *Cinchona ledgeriana* Moens and 40 of *C. pubescens* Vahl were planted in each chamber in March, 1945. Forty seedlings of *C. ledgeri-*

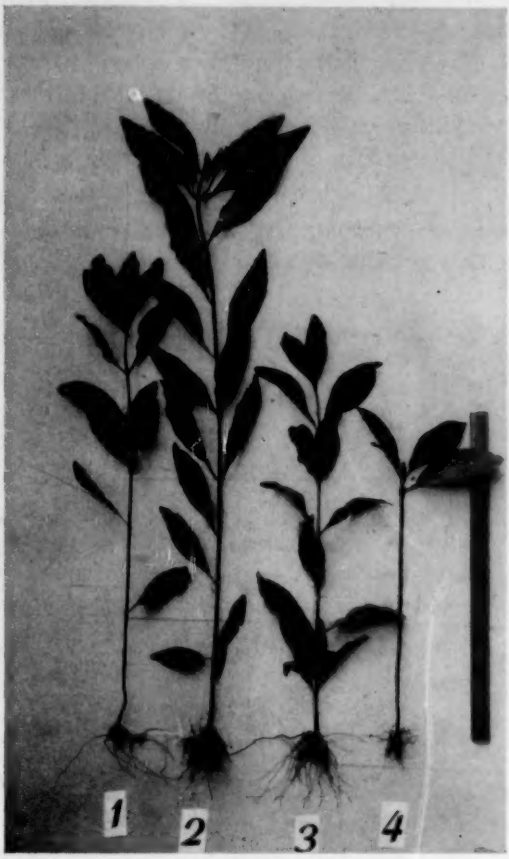

FIG. 3. *C. ledgeriana* seedlings grown under different temperature conditions: (1) warm chamber (70° to 80° F.), (2) medium chamber (65° to 75° F.), (3) cold chamber (60° to 70° F.), and (4) greenhouse bench where air temperature was usually on a higher level than that of the warm chamber. Note well-developed root system on typical plant from cold chamber (3) and sparse root system on plant from warm chamber (1). Stems and foliage of seedlings grown in the cold chamber were rigid; the tips and lower sides of the leaves had a reddish color.

ana were also placed in the greenhouse outside the chambers for comparison with the plants of the same species grown under controlled conditions. These were once-transplanted seedlings of uniform 4-inch height grown in a sheltered nursery at Maricao, Puerto Rico. Spacing in the greenhouse beds was 6 by 6 inches. Plants that died during the first weeks of the experiment

were replaced immediately in order to provide as nearly as possible a uniform stand.

On several occasions thrips and aphids appeared on the plants. Both were easily controlled by applications of derris dust containing 1% to 2% rotenone. Eradication of aphids was more difficult in the cold chamber than in the warm or medium temperature chamber.

Final observations and growth data were taken on February 25, 1946. The plants were removed from the beds and all excess soil washed from root systems. Total fresh and oven dry weights were recorded for each plant and separately for leaves, stems, and roots. Chemical analyses were made for total alkaloids and quinine in the stems and roots, by the method described by LOUSTALOT and PAGÁN (**3**).

Results

The two species of *Cinchona* responded quite differently to the four environments (table I and figs. 3 and 4).

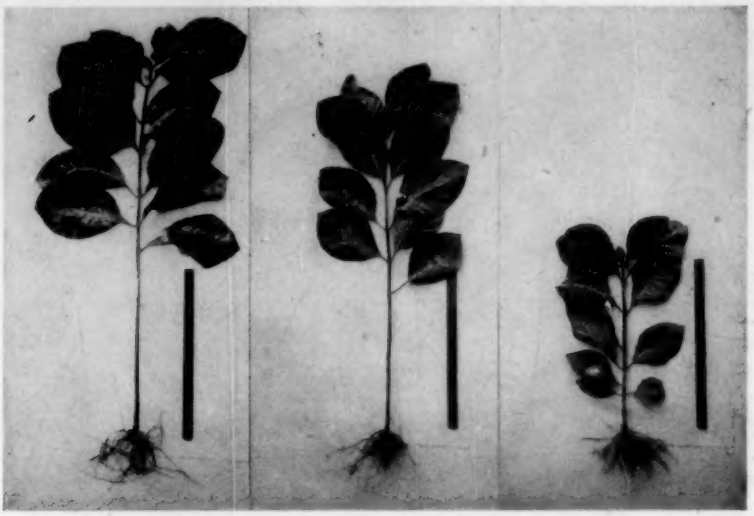

FIG. 4. Representative seedlings of *C. pubescens* grown under different temperature conditions. Left to right: warm chamber (70° to 80° F.), medium chamber (65° to 75° F.), and cold chamber (60° to 70° F.).

C. LEDGERIANA

In the warm chamber *C. ledgeriana* produced weak, slender stems and poor root systems. During the summer period many necrotic spots developed on the leaves. A more vigorous healthy growth was produced in the medium temperature chamber. In the cold chamber a stocky growth developed, characterized by thick stems, vigorous root systems, and undulate red tinged leaves. Growth was poorest in the greenhouse outside the chambers where temperatures averaged 5 to 10 degrees higher than in the warmest chamber. During the summer, large necrotic areas developed on the leaves

TABLE II

GROWTH DATA OF *C. ledgeriana* AND *C. pubescens* GROWN AT THREE TEMPERATURE LEVELS

ENVIRONMENT	*C. ledgeriana*					*C. pubescens*				
	FRESH WEIGHT*	DRY WEIGHT*	DRY MATTER†	HEIGHT*	SURVIVAL	FRESH WEIGHT*	DRY WEIGHT*	DRY MATTER†	HEIGHT*	SURVIVAL
	gm.	*gm.*	%	*cm.*	%	*gm.*	*gm.*	%	*cm.*	%
Warm	26.23	5.48	20.89	54.2	86.36	99.92	18.73	18.75	74.6	56.81
Medium	40.27	8.36	20.76	70.6	90.00	79.09	13.26	16.76	67.5	82.50
Cold	34.39	7.13	20.73	46.7	85.00	60.75	9.05	14.90	45.2	100.00

* Average per plant.
† Concentration of.

and subsequent defoliation left the plants almost leafless. Root development under these conditions was extremely poor (fig. 3).

Cinchona ledgeriana seedlings grown in the medium temperature chamber made the best growth as indicated by the average height and average fresh and dry weights per plant (table II). Seedlings grown in the warm chamber were taller, on the average, than those in the cold chamber, but they had an average fresh and dry weight less than the cold chamber plants. Plants grown in the greenhouse outside the chambers were inferior in growth to all plants grown in the chambers. These differences were all statistically significant at the 5% level and in some cases at the 1% level. There was no appreciable effect of temperature on the concentration of dry matter or on the percentage of plants surviving in the three chambers.

There was a definite effect of temperature on the shoot-root ratio; seedlings from the warm chamber had the highest ratio and those from the cold chamber the lowest (table III). The table also shows that the proportion of leaves by dry weight to the rest of the plant was not appreciably different in any of the treatments. Plants grown in the cold chamber, however, had

TABLE III

EFFECT OF TEMPERATURE ON PROPORTIONAL DRY WEIGHT OF LEAVES, STEMS, AND ROOTS OF *C. ledgeriana* AND *C. pubescens* SEEDLINGS GROWN AT THREE TEMPERATURE LEVELS

ENVIRONMENT	*C. ledgeriana*					*C. pubescens*				
	LEAVES	STEMS	ROOTS	SHOOT–ROOT RATIO	BARK ON STEMS	LEAVES	STEMS	ROOTS	SHOOT–ROOT RATIO	BARK ON STEMS
	%	%	%	%	%	%	%	%	%	%
Warm	43.61	43.97	12.42	7.01	20.44	33.74	48.85	17.41	4.75	20.84
Medium	41.38	45.45	13.17	6.58	23.10	41.18	43.14	15.68	5.39	20.66
Cold	42.91	40.67	16.42	5.10	29.00	42.88	35.24	21.88	3.56	23.06

a lower percentage of stem but higher percentage of bark than plants grown in either of the other chambers.

Under the conditions of this experiment there was little or no effect of temperature on the percentage of total alkaloids and quinine in the stems and roots of young *C. ledgeriana* seedlings (table IV). Figure 3 shows a

TABLE IV

TOTAL ALKALOID AND QUININE CONTENT IN ROOTS AND STEMS OF *C. ledgeriana* AND *C. pubescens* SEEDLINGS GROWN AT THREE DIFFERENT TEMPERATURES

ENVIRONMENT	*C. ledgeriana*				*C. pubescens*			
	TOTAL ALKALOIDS		QUININE SULPHATE		TOTAL ALKALOIDS		QUININE SULPHATE	
	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS
	%	%	%	%	%	%	%	%
Warm	2.79	5.20	0.80	1.80	3.12	5.24	1.0	1.45
Medium	2.69	3.38	0.50	2.10	2.97	4.45	0.7	1.30
Cold	2.92	4.21	0.93	1.90	2.60	3.10	0.9	0.70

representative *C. ledgeriana* plant from each chamber and also a seedling grown in the greenhouse bench where it was subjected to the natural fluctuations of greenhouse temperature.

C. PUBESCENS

The *C. pubescens* species of Cinchona is morphologically different from *C. ledgeriana* (table I and figs. 3 and 4). There was a direct correlation between growth and temperature (table II). The best and most vigorous growth as shown by height and weight measurements was produced in the warm chamber. The cold chamber produced short stocky plants with short internodes and excellent root systems. Plants from the medium temperature chamber were intermediate in size and vigor compared to those from the warm and cold chambers.

Percentage of survival for *C. pubescens* was inversely correlated with temperature, and all differences were of a high order of significance. In the warmer chambers competition due to the large leaves and close spacing of the plants apparently resulted in death of the weaker plants, while in the cold chamber where growth was slower and competition less, 100% survived. This is a contrast to the uniform survival in all chambers of the narrow-leaved *C. ledgeriana* where competition for light evidently was not a factor.

Unlike *C. ledgeriana* the concentration of dry matter in *C. pubescens* seedlings was directly correlated with temperature. The plants in the warm chamber produced the highest percentage of dry matter and those in the cold chamber the lowest. There was no significant temperature effect on shoot–root ratio (table III). The proportion of leaves to total plant on a dry weight basis was lowest in the warm chamber and highest in the cold

chamber, whereas the reverse was true with percentage of stems. As with *C. ledgeriana*, the percentage of bark on the stems was somewhat higher on seedlings grown in the cold chamber than on those grown in the warmer chambers.

The percentage of total alkaloids in stems and roots and quinine in the roots was directly correlated with temperature (table IV). The percentage quinine in the stems of plants grown in the medium temperature chamber was somewhat lower than that of plants grown in the warm and cold chambers.

Discussion

The results obtained in the experiment emphasize the importance of temperature as a factor influencing the growth and composition of Cinchona. A comparison of the responses of the two species to relatively small differences in temperature (5° to 10° F.) suggests that *C. pubescens* is better adapted to warmer temperatures than is *C. ledgeriana* which grows best at cool temperatures and is more exacting in its requirements. Of the two species, the *C. pubescens* was much more vigorous irrespective of temperature conditions. This may be due to its inherent ability to produce vigorous root systems under varied conditions. Development of the root systems of *C. ledgeriana* was adversely affected by higher temperatures.

Although plants of *C. ledgeriana* grown in the warm chamber made more linear growth than those from the cold chamber, the plants in the cold chamber had a greater average dry weight. The beneficial effect of the cold temperature in producing a higher percentage of bark for both species is offset by the smaller total dry weight produced under cool conditions.

Summary

1. *Cinchona ledgeriana* and *C. pubescens* were grown under three temperature controlled conditions ranging between 70° to 80° (warm), 65° to 75° (medium), and 60° to 70° F. (cold). In order to simulate natural conditions in each chamber, the night temperature was lowered 5° from October through March.

2. There was a marked difference in response of the two species to temperature. The *C. pubescens* seedlings grown in the warm environment had an average dry weight about twice that of plants grown in the cold treatment, while plants grown in the medium temperature environment were intermediate. There was a similar trend in height of plants.

The *C. ledgeriana* seedlings grew best in the medium temperature and were more exacting in their temperature requirements than *C. pubescens*.

3. There was no appreciable effect of temperature on percentage survival of *C. ledgeriana* seedlings whereas the percentage survival of *C. pubescens* was lower at the higher temperature level.

4. The shoot–root ratio of the *C. pubescens* plants was not significantly affected by temperature. The *C. ledgeriana* seedlings, however, were affected

by temperature; the lower the temperature the greater the roots in proportion to the shoots.

5. High total alkaloids and quinine in *C. pubescens* roots and stems seemed to be associated with high temperature and vigorous growth. There was no consistent trend in the *C. ledgeriana* seedlings in regard to these constituents.

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A COMPARISON OF PHOTOSYNTHESIS IN INDIVIDUAL PINE NEEDLES AND ENTIRE SEEDLINGS AT VARIOUS LIGHT INTENSITIES

PAUL J. KRAMER AND WALTER S. CLARK

(WITH THREE FIGURES)

Received July 5, 1946

It was found by KRAMER and DECKER (**5**) that loblolly pine seedlings have a relatively lower rate of photosynthesis at low light intensities than do certain species of competing hardwoods. Seedlings of dogwood, eastern red oak, and white oak attained a maximum rate of photosynthesis at an intensity equal to about one-fourth of full sunlight (2500 foot candles) and maintained an approximately constant rate at all higher intensities. Loblolly pine seedlings attained only about two-thirds their observed maximum rate at one-fourth of full sunlight, and the rate continued to increase to the highest intensity used, which was 9300 fc.

Various reasons might exist for the difference in behavior. There might be a fundamental difference in the photosynthetic mechanism of the pines and hardwoods, or the stomates of pine might require more light to open fully than do the stomates of hardwoods. Either situation could at least partly account for the observed difference. Consideration of certain published works suggested that mutual shading among the pine needles might account for the observed difference. HEINICKE and CHILDERS (**3**) found that individual, fully exposed leaves of apple attained their maximum rate of photosynthesis at one-fourth to one-third of full sun, but photosynthesis of entire trees was limited by light intensity up to full sunlight. This, they stated, indicated that a large part of the leaf area of an apple tree is too heavily shaded to carry on maximum photosynthesis except during the middle of very bright days. UHL (**7**) reported that in comparing the rates of photosynthesis of two-, three-, and five-needled species of pines the rate of photosynthesis per unit of photosynthetic tissue decreased as the number of needles per fascicle increased. Thus the rate of photosynthesis of two-needled pines was greater than that of five-needled pines, because the needles of the latter shade each other more. It was decided, therefore, to find whether the shading of some needles by other needles might be one cause of the relatively low rate of photosynthesis of loblolly pine seedlings exposed to light of low intensity.

Methods

The plants used were two-year-old loblolly pine (*Pinus taeda* L.) seedlings grown in pots out-of-doors in full sun. Fully matured needles of the current year's growth were used in all experiments to avoid variations in behavior caused by differences in age of needles. Mutual shading of the needles was prevented by arranging them in small leaf chambers (fig. 1).

The chambers were constructed of cellulose acetate sheet in such a manner that the needles of four fascicles could be inserted through the holes at each end and held perpendicularly to a light source and far enough apart that they could not shade each other. Details of their construction are shown (fig. 2). Each leaf chamber held a four-cm. segment of each of 12 needles, having a total leaf surface of about 2.3 cm., and three leaf chambers were attached to each seedling. The air temperature within each leaf chamber

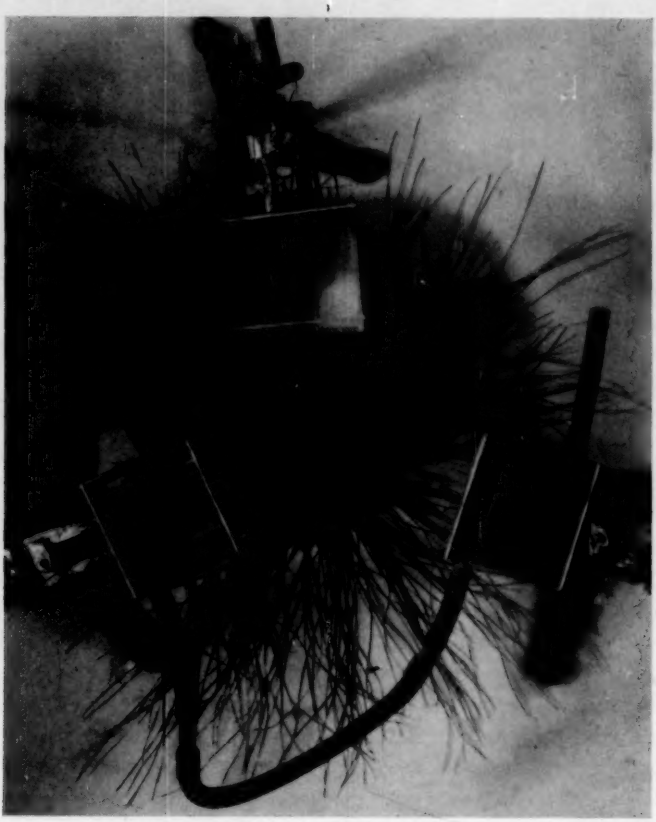

FIG. 1. View from above showing leaf chambers in place on loblolly pine seedling.

was maintained at 25° ± 1.5° C. by pumping cold water through the water jacket on the lower side of the chamber. Thermocouples were inserted in the chambers to measure the air temperature. The potted seedlings with their attached leaf chambers were placed in the constant temperature chamber described by DECKER (**2**) and kept at 26° ± 2° C. The light source was a battery of projector-type Mazda lamps which provided a maximum intensity of about 9300 fc below the top of the chamber. The leaf chambers were oriented with respect to the light source so they were always in the same position and at the same distance from it. The seven light intensities used were obtained by means of various combinations of shades made from

screen wire and cheesecloth. Each seedling was exposed to all seven intensities in one day, the sequence being randomized for each seedling to eliminate any systematic effect of sequence of intensities on the results. The period of exposure to a given light intensity was one hour, and a fifteen-minute adjustment period was allowed following each change in intensity. Fourteen plants were used, giving fourteen independent observations at each light intensity.

Apparent photosynthesis was measured by determining the difference in carbon dioxide content of an air stream before and after it had passed through the leaf chamber. The air streams from the three leaf chambers on a single plant were combined and passed through a spiral absorption tower

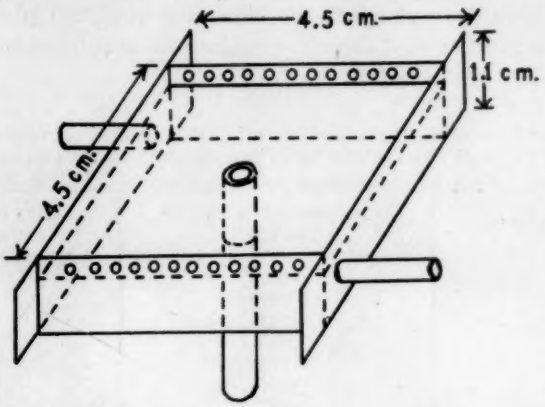

FIG. 2. Drawing of leaf chamber with cover removed. It is constructed of cellulose acetate sheet about 0.7 mm. thick, the pieces being cemented together with Duco cement. Cold water is circulated through the lower part of the chamber by means of the tubes projecting through the sides. Air enters through the same holes in the ends of the upper section which hold the needles, and it passes out through the tube in the center. Pieces of rubber band (not shown) were cemented to the upper edges of the leaf chamber to serve as a gasket for the cover which is a rectangular piece of cellulose acetate held in place by rubber bands slipped over the strips projecting at each end.

designed by the junior author as a modification of one described by LEACH, MOIR and BATHO (**6**). The tower contained 50 ml. of 0.01 N NaOH, and the change in concentration of the alkali caused by absorption of carbon dioxide was measured by means of the change in its electrical resistance. With the concentration of alkali, time interval and rate of air flow used in these experiments about 0.1 mg. of carbon dioxide could be measured. This represented about 2% of the total carbon dioxide absorbed by a tower. The rate of air flow was adjusted so that not over 15% of the carbon dioxide was removed by the pine needles, because it has been found that the rate of photosynthesis decreases rapidly when more than this is removed (**2**).

The volume of air passed over the pine needles was measured by means of calibrated flowmeters placed between the leaf chambers and the absorption towers. A simple automatic pressure regulating valve was devised to

maintain constant pressure on the vacuum line (1). Three absorption towers were operated simultaneously, two being used to measure the carbon dioxide content of air streams from two plants and the third to measure the carbon dioxide content of the air before it passed over the needles.

Results

The results of the experiments are summarized in table I and fig. 3. The rate per unit of leaf surface was calculated on the assumption that all samples of leaves had the same area, 2.3 sq. cm. If any error resulted from this assumption, it did not enter into the comparisons between light intensities because all such comparisons for each plant were made on the same leaf tissue. It is possible to compare the curve for apparent photosynthesis of individual pine needles with those for entire pine seedlings and entire hard-

TABLE I

RATES OF APPARENT PHOTOSYNTHESIS OF LOBLOLLY PINE NEEDLES AT VARIOUS LIGHT INTENSITIES. EACH VALUE IS THE AVERAGE OF 14 INDEPENDENT OBSERVATIONS ON 14 PLANTS

LIGHT INTENSITY	CO_2 ABSORBED PER 6.9 CM.^{2} PER HR.	RATE OF OBSERVED MAXIMUM
fc	*mg.*	%
375	0.277*	26.7
2250	0.721	69.6
3550	1.036	100.0
4350	0.934	90.2
6000	0.876	84.6
7550	0.882	85.1
9200	0.887	85.6

* The standard error of the difference between any two of these values is 0.103 and differences of twice this value are significant at the 5% level.

wood seedlings (fig. 3). There is an obvious difference between the behavior of entire pine seedlings and that of individual pine needles. The rate of photosynthesis of entire pine seedlings increased with increasing light intensity up to the brightest light used, but the rate of photosynthesis of individual, fully exposed pine needles increased only up to about one-third of the highest intensity used and then remained unchanged with further increase in light intensity. In this respect their behavior is similar to that of hardwood seedlings which are represented by the curve for eastern red oak (fig. 3). The data for entire pine and oak seedlings are from the work of KRAMER and DECKER (**5**).

Statistical analysis of the data for pine needles showed the increase in rate of photosynthesis from 330 to 3350 fc to be highly significant, but no significant difference exists between the rates at 3350 and 9200 fc. The rate of photosynthesis of entire pine seedlings was significantly higher at 9300 fc than at 3300 fc. There seems to be, therefore, a real difference between the behavior of the entire pine seedlings and that of the individual needles.

It seems possible that this difference in behavior can be attributed largely

to the greater amount of mutual shading which occurs among pine needles than among the leaves of most hardwood seedlings. No satisfactory method has been devised to measure mutual shading, but inspection of the leaf arrangement of dogwood and oak seedlings indicates that since the leaves form an umbrella-shaped mosaic they shade each other very little when illuminated from above. Pine needles, on the other hand, are arranged radially along several inches of stem and, therefore, shade each other. Pre-

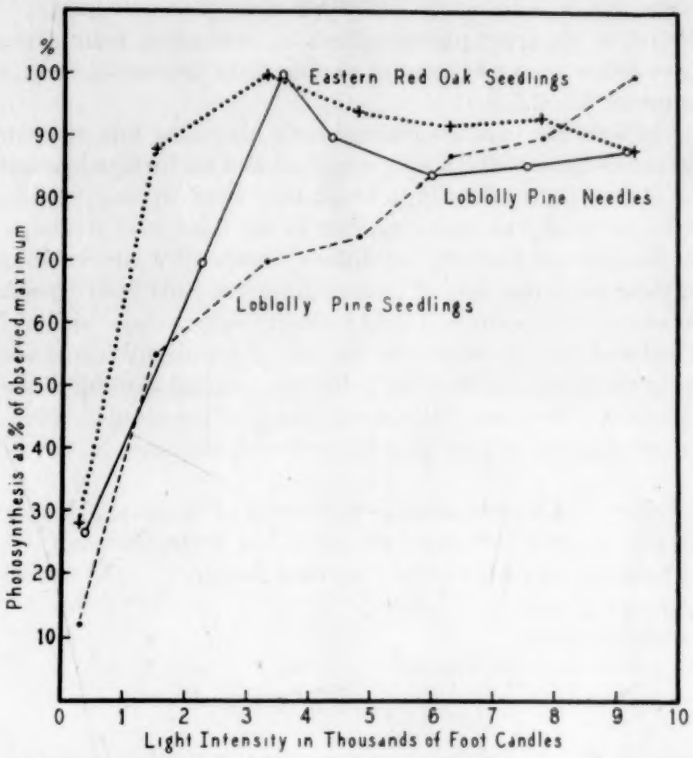

FIG. 3. Rates of photosynthesis of individual loblolly pine needles, entire loblolly pine seedlings, and eastern red oak seedlings at seven light intensities. The data for entire oak and pine seedlings are from KRAMER and DECKER (5).

sumably, the light intensity at the surface of all the needles averages only about 3000 to 3500 fc in full sun and much less in shade.

As the seedlings grow larger mutual shading probably becomes quite important, even in hardwoods, because an increasing proportion of the leaf surface is so located that it is shaded by branches and other leaves.

In view of these results it is possible that completely illuminated pine needles require no higher light intensity for maximum photosynthesis than do hardwoods. The failure of pine seedlings to carry on photosynthesis in the shade as rapidly as hardwood seedlings is probably the result of greater mutual shading in pine than in hardwoods. This does not exclude the possi-

bility that other factors contribute to the difference. It may be that instead of a direct effect of light on photosynthesis there is an indirect effect through the influence of light intensity on stomatal opening. There might even be a fundamental difference in the photosynthetic mechanism. It is hoped that these possibilities can be investigated at some future time, but it is believed that they are unlikely to be of major importance.

Summary

The rates of apparent photosynthesis of individual, fully exposed, loblolly pine needles were measured at various light intensities and a constant temperature of 25° C.

Fully exposed pine needles reached their maximum rate of photosynthesis at about one-third of full sunlight and showed no further increase in rate up to the highest light intensity to which they were exposed, 9200 fc. They behaved in essentially the same manner as the hardwood seedlings investigated by KRAMER and DECKER, but differed from entire pine seedlings which attained their maximum rate of photosynthesis at 9300 fc and reached only sixty per cent. of maximum at a light intensity equal to one-third of full sun.

It is believed that the relatively low rate of photosynthesis of entire pine seedlings in the shade results principally from mutual shading of the needles by one another. Because of the arrangement of the needles, there is much more mutual shading in pine than in hardwood seedlings.

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CHANGES IN FOOD RESERVES AND RESPIRATORY CAPACITY OF BINDWEED TISSUES ACCOMPANYING HERBICIDAL ACTION OF 2,4–DICHLORO–PHENOXYACETIC ACID[1]

FREDERICK G. SMITH, CHARLES L. HAMNER, AND ROBERT F. CARLSON

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Introduction

Widespread interest in the use of the synthetic growth regulator, 2,4-dichlorophenoxyacetic acid, as an herbicide has emphasized the need for increased knowledge of the mechanism of action of this and similar substances. Furthermore, the special significance of bindweed (*Convolvulus arvensis* L.) as an economic problem and the extensive study of its control by other means (**2, 3, 6, 19**) have made desirable the investigation of herbicidal action of the compound. HAMNER and TUKEY (**8**) and MARTH and MITCHELL (**11**) first investigated the application of 2,4-dichlorophenoxyacetic acid to bindweed, and TUKEY, HAMNER, and IMHOFE (**18**) studied in detail the histological changes preceding death. The most striking responses were the increased meristematic activity in the pericycle, phloem, and cambium regions, and the rapid disappearance of starch granules from the cortex of root and rhizome. An attempt was made in this investigation of the herbicidal action of 2,4-dichlorophenoxyacetic acid on bindweed to correlate changes in food reserves and respiratory capacity of underground structures with the gross symptoms, and the results of the investigation are reported.

Materials and methods

The plants and their treatments were similar to those used in the previous work at this Station (**8, 18**) and the experiments were carried out in the same fields. Three trials were made, two of which were analyzed in detail, the first in July and the second in August, 1945. At least two strips (each 6×1 ft.) of both treated and control plots including tops and underground parts to the depth of about 18 inches were harvested in the early afternoon, wrapped in damp towels, and taken immediately to the laboratory. After washing, the leaves, stems, and underground parts were separated, cut into lengths of $\frac{1}{2}$ to 1 inch, killed in a forced draft oven at 110° C. for 30 minutes, and dried overnight at 70° C. The dried samples were ground to 100-mesh in a Wiley mill and redried at 70° C. immediately before analysis. Aliquots of the fresh samples were dried at 110° C. for moisture determination, and aliquots of each ground sample also were redried at 110° C. for calculation on a dry weight basis.

Carbohydrate analyses were carried out by small-scale modifications of

[1] Journal Paper No. 683, New York State Agricultural Experiment Station, Geneva, N. Y., July 2, 1946.

earlier methods (**5, 9**). The technique, which has not previously been described, was developed primarily for use with tissue slices but was also found suitable for the present material. Samples of 100 mg., containing 1 to 10 mg. of soluble sugars and/or hydrolyzable polysaccharides, were extracted by refluxing for 20 minutes with 20 ml. of 80% ethanol in large test tubes (1 × 8 inch) with cold-finger condensers. The tubes were centrifuged, the supernatants decanted, and the residues reextracted in the same way. Two extractions were found to remove 98% to 99% of the soluble sugars. The combined extracts containing the sugars were concentrated on a steam bath to remove the ethanol and the final aqueous solutions of 4 or 5 ml. transferred to calibrated test tubes. Excess saturated neutral lead acetate was added, the volume made up to 10 ml., and the lead salts centrifuged down. The supernatants were then decanted into other tubes containing excess solid $K_2C_2O_4$ and the PbC_2O_4 centrifuged down. Aliquots of 2 or 4 ml. of the supernatants in large test tubes were acidified to about pH 4 with 10% acetic acid using methyl red; 4 drops of 0.1% invertase (Wallerstein Scales) were added and the tubes incubated 3 to 4 hours at 38° C. The volumes were then made up to 5 ml. with water and the reducing capacities measured with SOMOGYI's new copper-phosphate reagent (**17**) by titration with 0.005 N thiosulphate. The "total sugars" were expressed as mg. of glucose.

The estimation of the "starch-dextrins" fraction of the residues was carried out by a modification of the HASSID, MCCREADY, and ROSENFELS technique (**9**) using the copper-phosphate reagent above. It should be noted that grinding beyond 100-mesh or solubilizing by the HCl-ethanol method was not necessary under these conditions. The residues in the original tubes, with 4 ml. of water added, were first heated 30 minutes in a boiling water bath, then 1 ml. of pH 5.6, 0.1 M acetate buffer containing 0.25 N NaCl, 1 ml. filtered saliva (diluted 1:1), and a crystal of thymol were added. The hydrolysis limit was reached after less than 6 hours at 38° C., but the hydrolyses were usually run overnight. The contents of the original tubes were then transferred to calibrated test tubes, clarified, and analyzed as for the soluble sugars. The differences in reducing capacities between samples treated with boiled and unboiled enzyme solutions were expressed as starch based on a Kahlbaum starch standard. These differences were considered a measure of the "starch-dextrins" fraction which appears to be the principal polysaccharide reserve of bindweed roots (**2, 7**). This fraction plus "total sugars" constituted the "available carbohydrates."

Total nitrogen was run on a semi-micro scale by a technique based on that of MA and ZUAZAGA (**10**) using a selenium catalyst. No nitrates were found by the diphenylamine test.

Control and treated samples for respiration measurements from each of three plants were taken from rhizomes 4 to 6 inches below the soil line and from roots 8 to 12 inches lower. Freehand slices 0.3 to 0.5 mm. thick giving 100 to 200 mg. fresh weight per sample were washed in aerated, running tap water for 2 to 3 hours. The rate of oxygen uptake was determined sepa-

rately for root and rhizome slices of each plant in standard Warburg apparatus in 0.02 M pH 5.7 phosphate buffer and an oxygen atmosphere. Under these experimental conditions the rates were linear for at least an hour, were independent of slice thickness and shaking rate, and were proportional to the amount of tissue added. The least significant difference at the 5% level between samples for several experiments similar to that in table I was found to be about 0.10 microliters per hour per mg. fresh weight. Samples of the material from which the slices were cut were fixed and sections prepared as in the previous work (**18**).

TABLE I

CHANGES IN RESPIRATORY CAPACITY OF RHIZOME AND ROOT SLICES FOLLOWING TREATMENT WITH 2,4-DICHLOROPHENOXYACETIC ACID MEASURED IN WARBURG RESPIROMETER

DAY	OXYGEN PER HR./MG., FRESH WEIGHT*						RATIO OF TREATED TO CONTROL
	CONTROL TISSUE			TREATED TISSUE			
	RHIZOME	ROOT	MEAN	RHIZOME	ROOT	MEAN	
	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	
3	0.36	0.32	0.34	0.58	0.39	0.49	1.44
6	0.34	0.27	0.31	0.59	0.57	0.55	1.78
10	0.28	0.31	0.30	0.61	0.64	0.63	2.10
14	.....	.....	0.33	.....	.....	0.55	1.67

* Average of three determinations.

Results

The gross response to treatment agreed closely with that observed in 1944 (**18**) though the sequence of symptoms was somewhat slower. On the second day the leaves were severely folded and most stems were limp. By the fourth or fifth day some lower leaves were chlorotic and a few had necrotic areas. By the sixth or seventh day the rhizomes showed definite external signs of proliferation and both stem and leaves had necrotic areas. By the tenth day swelling of the rhizomes and roots was pronounced and 10% to 20% of the leaves and stems were dead. On the fourteenth day so much of the tops were dead that only root samples were taken for analysis. It should be noted that as far as possible no dead tissue was included in these samples. Histological sections of rhizomes and roots showed similar changes to those reported the previous year (**18**).

Changes in carbohydrate and nitrogen fractions following treatment in the July samples only are given (table II) since the August samples yielded similar results. Expression on a fresh weight instead of dry weight basis gave the same general picture with somewhat increased ratios of root constituents. However, fresh weight determinations by the tenth and fourteenth days were of limited significance because of wilting in the tops and softening of the root cortex. As early as the second day there was evidence of carbohydrate mobilization in the leaves. Total sugars reached a peak by the fourth day and then returned to the control level while the starch-

TABLE II

CHANGES IN COMPOSITION OF BINDWEED FOLLOWING TREATMENT WITH 2,4-DICHLOROPHENOXYACETIC ACID GIVEN AS PERCENTAGE OF DRY WEIGHT

DAY	TISSUE	DRY WEIGHT		TOTAL SUGARS			STARCH-DEXTRINS			TOTAL AVAILABLE CARBOHYDRATES			TOTAL NITROGEN		
		C*	T†	C*	T†	T/C‡	C*	T†	T/C‡	C*	T†	T/C‡	C*	T†	T/C‡
		%	%	%	%		%	%		%	%		%	%	
0	Leaf	17.5	.....	4.80	.....	.....	2.55	.....	.....	7.35	.....	.....	4.78	.....	.....
2		17.7	17.4	5.90	8.15	1.38	4.88	4.45	0.92	10.78	12.60	1.17	4.57	4.80	1.05
4		18.2	17.2	3.60	7.35	2.04	3.18	3.72	1.17	6.78	11.07	1.64	5.08	4.41	0.87
7		17.2	18.6	5.60	8.35	1.49	4.10	2.88	0.70	9.70	11.23	1.16	4.76	4.11	0.86
10		17.0	17.8	3.45	3.65	1.06	3.28	1.18	0.36	6.73	4.83	0.72	5.00	3.31	0.66
0	Stem	17.9	.....	5.70	.....	.....	0.70	.....	.....	6.40	.....	.....	2.25	.....	.....
2		17.0	17.3	7.35	10.95	1.49	0.60	1.10	1.84	7.95	12.05	1.52	2.23	2.52	1.13
4		19.0	20.2	7.35	11.20	1.52	0.72	1.34	1.86	8.07	12.54	1.56	2.50	2.72	1.09
7		17.7	18.7	6.70	9.80	1.46	0.70	0.80	1.14	7.40	10.60	1.43	2.45	3.19	1.30
10		17.8	18.1	6.75	5.05	0.75	0.87	0.55	0.63	7.62	5.60	0.74	2.53	3.40	1.35
0	Rhizome-root	21.2	.....	6.85	.....	.....	3.48	.....	.....	10.33	.....	.....	1.50	.....	.....
2		19.4	19.3	6.55	8.20	1.25	5.63	5.65	1.00	12.18	13.58	1.12	1.79	1.60	0.90
4		22.1	19.2	8.15	11.50	1.41	5.31	4.30	0.81	13.64	15.08	1.10	1.73	1.97	1.14
7		19.6	18.3	6.85	11.60	1.70	4.02	2.48	0.62	10.87	14.08	1.30	1.87	2.13	1.14
10		20.9	18.1	6.75	9.45	1.40	6.30	2.45	0.39	13.05	11.90	0.91	1.78	2.48	1.39
14		23.9	.....	6.75	7.65	1.15	6.80	2.25	0.33	13.55	9.90	0.73	1.70	2.67	1.57

* Control.
† Treated.
‡ Ratio of treated to control.

dextrins fraction after a less significant rise dropped to one-third the control level and total nitrogen underwent a steady decrease. Changes in total sugars and starch-dextrins in the stem were similar to those in the leaves though neither the initial increase nor final decrease was so large. The nitrogen content, however, increased significantly above the controls, as would be expected from the histological evidence of meristematic activity in the stem (**18**). There was, likewise, a rise in total sugars in the roots and rhizomes of treated plants reaching a maximum about the seventh day in this case and again followed by a decrease. The starch-dextrins fraction, on the other hand, steadily dropped after the second day reaching one-third the control level on the fourteenth day. As in the stems the total nitrogen began to increase by the fourth to seventh day paralleling the increasing meristematic activity.

The changes in the carbohydrate and nitrogen fractions in bindweed treated with 2,4-dichlorophenoxyacetic acid were in many ways similar to those observed by MITCHELL and others (**1, 13, 14, 15**) in kidney bean seedlings treated with indoleacetic acid, naphthaleneacetic acid, and related compounds, and more recently by MITCHELL and BROWN (**16**) in annual morning-glory treated with 2,4-dichlorophenoxyacetic acid. The initial rise in total sugars in treated leaves before any corresponding drop in the starch-dextrins fraction seemed to indicate a temporary increase of photosynthesis over utilization. The trend was reversed, however, after the first four days and was followed by a drop in both carbohydrate fractions and total nitrogen as the leaves became chlorotic and wilted. If leaf metabolism was stimulated in any way at the outset, it was soon disrupted and became essentially catabolic. On the other hand, the metabolic stimulation of the rhizomes and roots seemed to be essentially anabolic: the histological evidence (**18**) of meristematic activity and increase in total nitrogen was sharply contrasted with the behavior of the leaves. While leaf reserves were quickly mobilized and translocated or consumed with a decrease in leaf nitrogen content, the mobilization in the rhizome and roots was marked by increased nitrogen content, synthesis of protoplasm, and cell division.

Further evidence of stimulated metabolism in treated rhizomes and roots was apparent in the typical set of respiratory measurements (table I). There was a significant increase in respiratory rates of treated tissues over control tissues as early as the third day in the rhizome and the sixth day in the lower root tissues. The increased ratio reached a maximum by the tenth day and declined by the fourteenth. Histological sections from the same samples, however, showed little if any cell division by the third day, indicating, as might be expected, that increased respiratory activity preceded increased cell division. The drop in rate of treated slices by the fourteenth day was probably due to the already evident disorganization and rupture of the cortical regions.

It is also interesting to compare the results of treatment with 2,4-dichlorophenoxyacetic acid with those of other bindweed control methods which have

often been interpreted in terms of their effect on root reserves. BAKKE, GAESSLER, and LOOMIS (**2**) concluded that in both chlorate treatment and fallowing the death of the roots was correlated with reduction of these reserves, though it did not occur so rapidly as in the case of 2,4-dichlorophenoxyacetic acid treatment. These treatments also differed from the present in causing no increase either in total sugar or nitrogen fractions or, apparently, in cell division. The workers suggest, however, but without direct evidence, that the rapid reduction in polysaccharide reserves with no corresponding rise in soluble sugars following chlorate treatment was due to stimulated respiration rather than to interference with photosynthesis.

TUKEY, HAMNER, and IMHOFE (**18**) proposed on the basis of histological changes four possible mechanisms contributing to the herbicidal action of 2,4-dichlorophenoxyacetic acid: (1) chlorophyll depletion in the leaves diminishing food production, (2) phloem proliferation in the vascular bundles interfering with translocation of food, (3) increased respiratory activity depleting food reserves, and (4) disorganization and rupture of rhizome and root cortex leading to invasion of soil pathogens and decay. The present analytical evidence, though admittedly limited, may throw some light on the mechanisms. The first was supported indirectly by the evidence for decline in carbohydrate production after the fourth day as the leaves became chlorotic. Similarly, the observed increases in respiratory capacities of rhizome and root slices tended to support the third mechanism. It seemed clear, however, that the reduction in available carbohydrates whether by diminished production or increased utilization was not the principal cause of death. The reduction was considered neither soon enough nor large enough to account, in itself, for the rapid death of the tops and partial disintegration of the roots by the tenth to fourteenth days. In the case of the second mechanism there was no specific evidence in the analyses for interference with phloem function though the possibility is an intriguing one and is given further consideration later. The fourth mechanism may be of major importance in the ultimate death of underground structures, but it is definitely secondary to the changes investigated and may even be a result of them.

The altered respiratory capacity and increased meristematic activity may be significant clues to other specific physiological abnormalities in the treated plant rather than to simple depletion of food reserves. Several lines of evidence suggest that the phloem may be especially involved: (1) rapid mobilization of food reserves to treated regions, presumably through the phloem, is characteristic of many responses to growth regulators (**13, 14**); (2) phloem is particularly sensitive to histological reaction to the agents (**4, 18**); (3) phloem activity in solute translocation (at least in the sieve tubes) is reported to require respiratory energy (**12**); and (4), it was indicated, though not proved by the study, that the phloem undergoes marked changes in respiratory activity following treatment. One may speculate, therefore, that the treatment caused changes in the respiratory mechanism of phloem

cells which under certain conditions, perhaps low concentrations, accelerated food translocation and under others, perhaps when cell division resulted, impeded it. Whether or not the above hypothesis be true, the analytical results seem to indicate that the herbicidal effect of 2,4-dichlorophenoxyacetic acid on bindweed is probably not due primarily to depletion of food reserves as was the case in earlier methods of control, but is largely the result of other physiological disturbances.

Summary

The mechanism of the herbicidal action of 2,4-dichlorophenoxyacetic acid on bindweed was further investigated by determining the changes in carbohydrate and nitrogen fractions and respiratory capacities of treated and control tissues. Small-scale methods for total sugars and the starch-dextrins fraction were described incidentally.

During the first few days the treatment caused rapid increases in total sugars in leaves, stems, and underground parts, and the increases were followed by decreases to control levels. The starch-dextrins fraction decreased in all three, reaching one-third the control levels in leaves, roots and rhizomes by the tenth day. Total nitrogen, on the other hand, decreased steadily in the leaves but increased in both stems and underground structures. The changes in nitrogen content of treated tissue appeared to be correlated with increased meristematic activity. Further evidence of stimulated metabolism was found in the increased respiratory activities of treated rhizome and root slices.

Comparison of these results with the effect of other growth regulators on bean seedlings showed striking similarities in the general mobilization of carbohydrates and nitrogen. Comparison with the effect of chlorate treatment on bindweed root reserves also showed certain similarities but did not indicate that reserve depletion as such was the chief mechanism of herbicidal action in the hormone treatment.

The bearing of the analytical results on the mechanisms of herbicidal action proposed by TUKEY, HAMNER, and IMHOFE (**18**) was discussed, and it was suggested that a specific mechanism of herbicidal action of 2,4-dichlorophenoxyacetic acid might be found in the interference with phloem function.

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STUDIES ON THE RELATIONSHIP BETWEEN THE RATE OF INFECTION OF COTTON SEEDLINGS BY *PHYMATOTRICHUM OMNIVORUM* AND THE AVAILABLE OXYGEN SUPPLY[1]

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(WITH FOUR FIGURES)

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In a previous paper (**5**) it was suggested that growth of and infection by the cotton root-rot organism, *Phymatotrichum omnivorum,* were both dependent on the oxygen supply available to the organism, since infection did not occur unless the organism was growing near the surface of the medium. The work of MOORE (**7**) has shown that for agar cultures the optimum oxygen concentration over the culture is that of the atmosphere, or about 21%. When the cultures are submerged, growth is apparently directly correlated with oxygen concentration in the medium. Under ordinary conditions the oxygen concentration in the lower part of a liquid culture approaches zero.

It was noted in previous experiments (**5**) that floating inocula of *P. omnivorum* grew much more rapidly than did those which were submerged. Infection of cotton seedlings was correlated with these conditions in such a way that when the culture was submerged, there was a delay in infection until the fungus had grown to the surface. Infection by surface cultures was much more rapid than by submerged cultures. Such a correlation led to the hypothesis that the oxygen concentration available to the fungus under these conditions was the limiting factor in infection. Work reported in this contribution was designed to prove this hypothesis.

Methods

The culture media, methods of inoculation, and seed treatment employed have been reported in a previous paper (**5**). The obvious approach to the problem would be to control the aeration of the culture medium so as to maintain uniform oxygen concentration throughout. It was found that simple aeration devices could not be employed with *P. omnivorum* cultures, since even the smallest bubbles from a sintered glass aerator cause sufficient disturbance of the culture to impair normal growth. The root-rot organism is very sensitive to mechanical disturbance when grown in liquid media, so that the growth rate is lessened considerably by shaking or even handling. It was necessary, therefore, to resort to other devices to accomplish aeration.

The aeration device used is shown in Tube T, figure 1. Half-inch cellophane dialysis tubing was cut into nine-inch lengths. A glass U-tube with an inlet (A) and an outlet (B) aperture was bent as shown. The gas

[1] This investigation is a contribution by the Clayton Foundation, Cotton Investigation and Research, The University of Texas, Austin, Texas.

entered the cellophane bag through opening (F) and returned to the glass tube through opening (D). The outlet (B) opening was reduced in diameter so that a slight pressure would result, thereby maintaining the cellophane bag (C) distended. The dialysis tubing was pushed around the glass tube and sealed by means of household cement (cellulose nitrate). The tubing was long enough so the seals would always be above the level of the

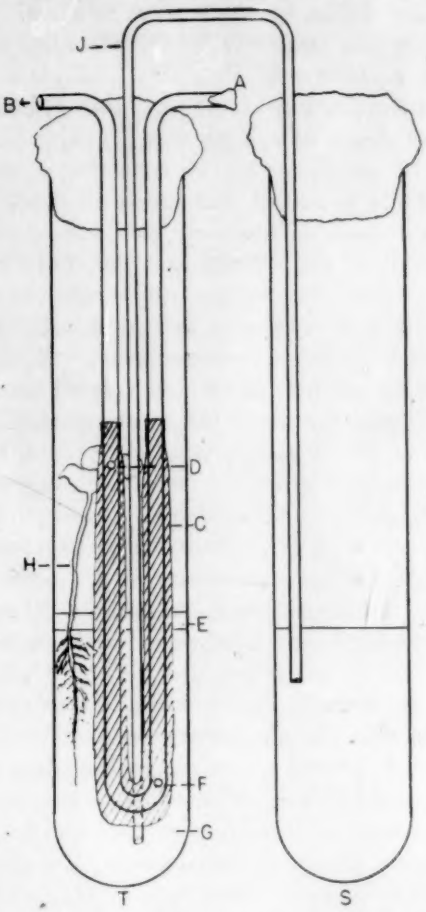

FIG. 1. Apparatus for the double culture of *Phymatotrichum omnivorum* and the cotton plant.

culture solution. An imperfect seal would thus not disturb the culture by allowing bubbles to pass up through it. The opening of the inlet tube was plugged with cotton so the air would be sterilized by filtering before it passed through the bag. The inlet tubes were attached to a source of compressed air, and a pressure of three cm. of mercury was maintained during an experiment. The test tube, with aeration device and medium, was sterilized in the autoclave for 20 minutes at a pressure of 15 pounds. During

sterilization and until the air pressure was applied, the tubes with the cellophane bags were kept above the surface of the medium to prevent the medium from passing inside the bags. When the air was attached, the pressure inside was sufficient to prevent the inward flow of the liquid medium.

The efficiency of the aeration apparatus was tested in order to determine the rate of oxygen absorption by the culture solution. Oxygen dissolved in the culture solution was measured by the WINKLER method. The procedure was modified according to VAN DAM (12). A 5-cc. hypodermic syringe was used as a collecting tube. A 3-inch spinal puncture needle was attached to it by means of sealing wax. The whole collecting device was simple enough for sterilization. In collecting a sample, the syringe with the attached needle was filled with the $MnSO_4$ solution, the plunger was forced completely down so that only the bore of the needle and the space between the wall of the syringe and the plunger were filled with $MnSO_4$. This amount was sufficient for an analysis. The tip of the needle could be easily sterilized, if necessary, so that it could be inserted into a sterile culture without danger of contamination. A sample for analysis was obtained by pushing the plug of the test tube to one side, inserting the syringe and needle so that the tip of the needle reached the bottom of the tube, and withdrawing a 5-cc. sample into the syringe, mixing thoroughly with the $MnSO_4$ during the process. The other reagents were added by drawing them into the syringe and shaking thoroughly. At no time was the sample in direct contact with air. Titration was carried out using N/10 sodium thiosulphate in a 0.2-cc. automatic micro burette.

It was necessary to test several materials as seals (E) against the permeation of oxygen before selecting the conditions for the experiments. Paraffin proved too brittle to allow the penetration of the needle for drawing off the oxygen test sample. Paraffin oil was unsatisfactory due to the solubility of oxygen in paraffin oil. Paraffin-paraffin oil mixtures or paraffin-petrolatum mixtures, however, proved efficient as seals against oxygen. The data from one of these experiments are presented (fig. 2 and table I).

Twelve tubes of mineral solution were set up. The first two were covered with 10 cc. of a mixture containing equal amounts of paraffin, petrolatum, and paraffin oil. The second two tubes were covered with 10 cc. of a mixture containing equal amounts of petrolatum and paraffin oil. The third two tubes were covered with one-half petrolatum and one-half paraffin. Tubes 7 and 8 were similar to 1 and 2 except that they were aerated; tubes 9 and 10 duplicated 3 and 4 but were aerated; tubes 11 and 12 duplicated 5 and 6 but were aerated. After first being sterilized for 20 minutes in an autoclave, all tubes were placed in a water bath at 30° C. Readings were begun as soon as the temperature of the tubes had reached equilibrium with the bath. The results are shown in table I and figure 2.

Any combination of materials used proved to be an adequate seal, as shown by tubes 1, 2, 3, 4, 5, and 6. The cellophane tubing permitted oxygen

TABLE I

EFFECT OF SEAL ON ABSORPTION OF OXYGEN FROM AIR

HOURS	AVERAGE OXYGEN IN SOLUTION					
	PARAFFIN-PETROLATUM-PARAFFIN OIL SEAL		PETROLATUM-PARAFFIN OIL SEAL		PETROLATUM-PARAFFIN SEAL	
	(TUBES 1 & 2) UNAERATED	(TUBES 7 & 8) AERATED	(TUBES 3 & 4) UNAERATED	(TUBES 9 & 10) AERATED	(TUBES 5 & 6) UNAERATED	(TUBES 11 & 12) AERATED
	%	%	%	%	%	%
0	0.290	0.300	0.291	0.291	0.295	0.304
3	0.310	0.363	0.304	0.342	0.287	0.340
6	0.318	0.397	0.291	0.373	0.295	0.376
11	0.321	0.414	0.300	0.441	0.296	0.423
22	0.335	0.433	0.308	0.461	0.315	0.455

to pass through in a short time, as shown in tubes 7, 8, 9, 10, 11, and 12. All the oxygen in these solutions passed through the cellophane, since the ture of petrolatum and paraffin was easier to manipulate in making the seal and in withdrawing the sample, and since it proved to be as efficient a seal as any, it was used as the seal in all other experiments. Since glucose must solutions were covered with the same seals as the preceding tubes. A mix- be present in the culture medium in relatively high concentration for optimum growth of Phymatotrichum, several experiments were conducted to test the oxygen uptake of high glucose media.

The results of all experiments using mineral solution and mineral solution plus glucose have been summarized (table II and fig. 3). It will be noted that the solutions were saturated with oxygen between 12 and 18 hours of aeration. The seal, however, apparently allowed a small amount of oxygen to permeate the solution, but a consistently low level was main-

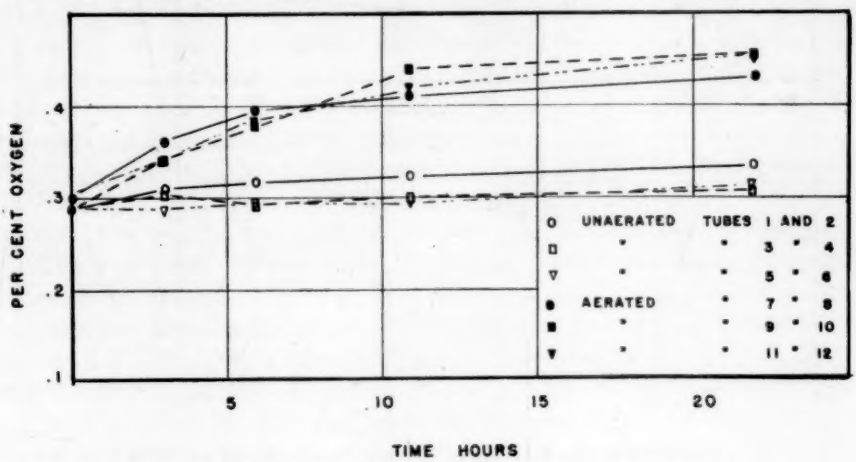

FIG. 2. Effect of seal on absorption of oxygen from air.

TABLE II

OXYGEN UPTAKE IN AERATED AND UNAERATED MINERAL AND MINERAL PLUS GLUCOSE SOLUTIONS

HOURS	OXYGEN IN SOLUTION			
	MINERAL SOLUTION		GLUCOSE SOLUTION	
	UNAERATED	AERATED	UNAERATED	AERATED
	%	%	%	%
0	0.238	0.244	0.164	0.135
2	0.264	0.321	0.170	0.212
4	0.260	0.387	0.175	0.220
8	0.265	0.445	0.185	0.360
12	0.282	0.471	0.195	0.370
24	0.317	0.500	0.190	0.401

tained in the unaerated media. The mineral solution and the sugar solution values for one set of conditions parallel each other closely. The sugar solution, however, is consistently lower in oxygen concentration than the mineral solution. By averaging all the values at any one time for both mineral solution and sugar solution and comparing one with the other, an average difference of 0.10% was obtained. If all the values for the sugar solution (table II) are raised by this amount, the sugar solution curve is very similar in shape and position to the mineral solution curve (fig. 3). This difference is apparently due to the difference between the solubility of oxygen in the mineral salt solution and its solubility in the medium containing 4% glucose. This difference in solubilities is confirmed by published reports (**9**). The

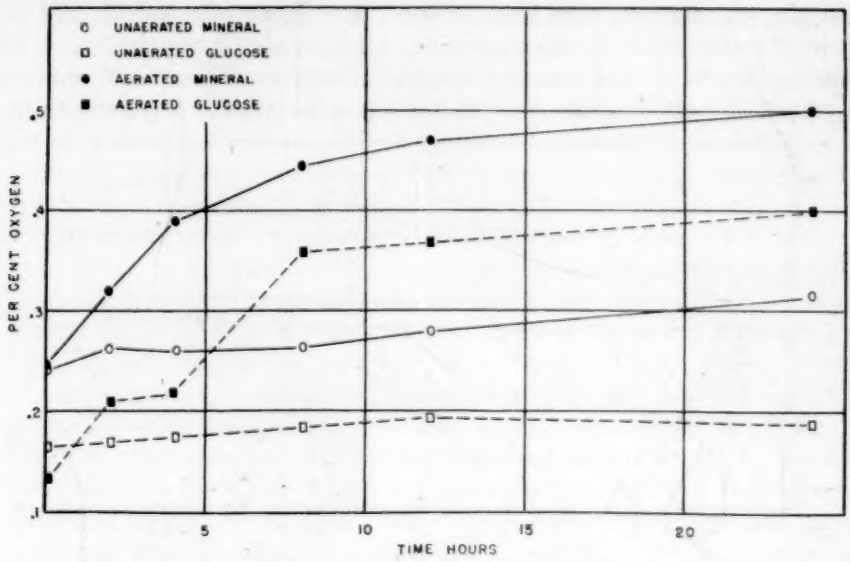

FIG. 3. Oxygen uptake in aerated and unaerated mineral and mineral plus glucose solutions.

values for the amount of dissolved oxygen in the mineral solution and in distilled water when saturation is reaching at 30° C. are comparable with those given in chemical handbooks for amount of oxygen from air dissolved in water.

Experimental results

Large test tubes were inoculated with *P. omnivorum* by transferring 10-cc. aliquots of an actively growing week-old liquid culture into 40 cc. of fresh medium for the test incubation. Group 1 consisted of eight tubes sealed with paraffin-petrolatum and aerated by means of the cellophane bags; group 2 consisted of eight tubes unsealed; and group 3 consisted of eight tubes sealed but not aerated. The tubes were incubated in a water bath at 30° C. for 17 days. At the end of the incubation period the experiment was dismantled and the mass of mycelia from each tube carefully rinsed in distilled water, blotted, and placed in watch glasses. Each lot was dried to constant weight (18 hours at 60° C.) and then weighed (table III).

TABLE III

WEIGHT OF DRIED CULTURE OF *P. omnivorum* GROWN UNDER DIFFERENT OXYGEN CONCENTRATIONS

GROUP	NUMBER	MEAN WEIGHT	STANDARD DEVIATION
		mg.	
Inoculum control	8	10.7	3.2
1	8	151.6	2.3
2	7	97.9	1.9
3	5	11.4	1.0

The weight of the inoculum was determined by washing, drying, and weighing eight inocula by the same methods as were employed in handling the test cultures.

Similar results were obtained on several repetitions of this experiment. It is apparent that a supply of oxygen is necessary for growth of the fungus. Growth in the sealed tubes was slight, amounting only to 0.7 mg. when the weight of the original inoculum was subtracted from the average of group 3. The slight growth in the sealed, unaerated tubes might have been due to a small amount of oxygen carried in on the inoculum and/or the slight seepage of oxygen through the seal. In the unsealed tubes, growth amounted to 87.2 mg. of dry mycelial weight. This is 125 times more than in the sealed tubes. Oxygen in this case was obtained only at the surface of the cultures by diffusion from the atmosphere. Since the inside diameter of the tubes was 3.6 cm., the surface was 10.17 sq. cm. Growth in the aerated tubes resulted in 141 mg. dry weight of mycelia. This is 1.6 times more than occurred in group 2. The surface of the cellophane bags which were employed in group 1 was approximately 15.5 sq. cm. It appears from this that growth of the fungus is correlated with the area of the membrane, or the

TABLE IV

MEAN WEIGHT OF MYCELIUM PRODUCED IN VESSELS ALLOWING DIFFERENT AREAS OF EXPOSURE TO AIR

VESSEL		AREA	MEAN WEIGHT	STANDARD DEVIATION
FLASK	TEST TUBES			
cc.		*sq. cm.*	*mg.*	
500	.....	78.5	59.3	6.6
250	.....	51.5	48.2	5.3
125	.....	34.2	41.3	6.6
	Large	9.6	27.3	0.0
	Small	3.8	16.6	1.1

effective surface across which oxygen can diffuse. Diffusion of oxygen through liquid cultures is not rapid enough to supply the needs of a rapidly growing submerged culture.

To test the relationship between the surface area exposed to air and the growth of the fungus, an experiment was set up wherein the vessels in which the fungus was grown allowed various surface areas of media to be exposed to atmospheric oxygen. Group 1 consisted of five 500-cc. Erlenmeyer flasks, group 2 of five 250-cc. flasks, group 3 of five 125-cc. flasks, group 4 of five large test tubes, and group 5 of five small test tubes. 40 cc. of medium were placed in each vessel. Each was inoculated with a uniform inoculum from an agar culture of *P. omnivorum*. At the end of a two-week incubation period the fungal mats which had formed on the surface of the liquid media were rinsed in distilled water, blotted, and dried as before. The mean weights of these mats with the area of the vessels in which they were grown are given (table IV and fig. 4).

Since it has been demonstrated that growth of the root-rot organism is dependent on the amount of oxygen available to it, the effect of oxygen

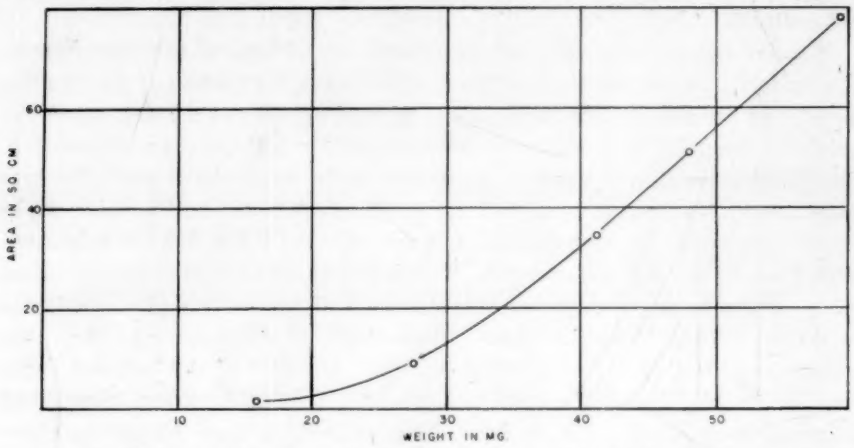

FIG. 4. Mean weight of mycelium produced in vessels allowing different areas of exposure to air.

concentration on the infection by this organism as measured by wilting of cotton seedlings was then studied. A slight modification in the apparatus was necessary since some liquid was removed from under the paraffin-petrolatum seal by the transpiring cotton plant. A second tube (S) was connected to the experimental tube by a siphon (J). As the liquid was removed from the first tube it could be replaced aseptically, and without breaking the seal, by merely raising the second tube to the necessary height. This culture unit is shown (fig. 1). The entire unit was set up complete, except for the seal, and sterilized in the autoclave. Precautions were taken to avoid breaking the siphon during the following manipulations. The experimental tubes were inoculated with uniform-sized inocula from a two-week-old agar culture of *P. omnivorum*. The inocula were purposely placed at the bottom of the tubes. A sterile paraffin-petrolatum seal was poured over the surface of the medium and around the tubing which passed through it. When the seal had solidified, a small opening was made at one side, and

TABLE V

DAYS REQUIRED FOR WILTING OF COTTON SEEDLINGS UNDER DIFFERENT OXYGEN CONCENTRATIONS

GROUP	NUMBER	MEAN NUMBER OF DAYS	STANDARD DEVIATION
Sealed and aerated	8	11.6	2.2
Unsealed	8	14.8	2.9
Sealed and unaerated	8	16.5	2.3

a sterile germinating cottonseed placed in it in such a way that the seed rested on the surface of the seal with the hypocotyl extending down into the medium. A small piece of sterile cotton was wrapped around the hypocotyl at the base of the seed to act as a wick in order that humidity conditions would be favorable for the opening of the cotyledons.

Three groups of cultures were set up: group 1 consisted of eight experimental tubes as just described with the aerating device and siphon; group 2 consisted of eight unsealed and unaerated tubes; and group 3 consisted of eight tubes in which there were no aerating devices, but in which the medium was covered with the seal. There were thus three different oxygen concentrations under which the cotton root-rot organism, and the cotton plant could be grown. The number of days for the various seedlings to wilt as a result of an attack by the fungus was noted (table V).

Isolations of the organism from the infected roots were performed in several cases, but this procedure was unnecessary since in all cases the fungus could be observed in the roots with no difficulty, and wilting was manifest within two or three days after the onset of attack. Since the plants were growing in liquid media and the aerial portions of the plant were growing in an atmosphere of high humidity, wilting was somewhat slower than it would have been in the field or even in potted plants in the greenhouse.

Discussion

The results obtained from the double culture of cotton plants and Phymatotrichum in liquid culture under controlled conditions have enabled the isolation of one factor of the environment for study. It has been demonstrated that cotton seedlings can be killed by the root-rot organism in liquid culture and that infection can be limited by control of oxygen concentration in the medium. The susceptibility of cotton seedlings to the organism under the conditions of the reported experiments indicates little justification for the frequently expressed opinion that young cotton plants are resistant to root rot. BLANK (**2**) has reported a differential susceptibility to root-rot infection between young and old seedlings and suggested the possibility that carbohydrate accumulation in seedling tissues enhanced the infectivity of the organism. WATKINS (**13, 14**) employing pure cultures, studied infection of cotton seedlings in media of high carbohydrate levels and demonstrated early kill not only of cotton seedlings but also of "immune" plants, *e.g.*, corn. EATON AND RIGLER (**3**), however, tested the carbohydrate hypothesis of root-rot infection and concluded that in soil cultures the carbohydrate concentration in root tissue does not affect infection. The Phymatotrichum cultures reported here were grown in media containing four per cent. glucose; therefore, according to BLANK AND WATKINS, conditions for infection were favorable. Under these conditions, it was possible to limit infection rate solely by altering available oxygen; consequently, it may be concluded that oxygen is one of the major factors in growth and infection.

The artificiality of laboratory conditions precludes a ready translation of these results to an interpretation of field observations. However, such an attempt should be made, recognizing the limitations of such an extrapolation. Under field conditions infection of cotton seedlings has been observed infrequently; ROGERS (**8**) expressed the belief that field kill occurs only in well-advanced plants. Soil temperature and moisture content during the seedling growing period have been suggested as limiting factors in infection as well as the reduced probability of contact between roots and organism at that time of the year.

That oxygen can play an important role in the complexity of factors influencing infection in the laboratory suggests the possibility of a similar effect of oxygen in the field. The high moisture content of the soil in the spring may limit the oxygen supply to rapidly growing organisms in the lower soil strata, since it has been demonstrated (**5**) that diffusion through a liquid medium is not sufficient to maintain a submerged culture at high infective rate of growth.

STREETS (**10**) reported that flooding experiments in the Yuma Valley demonstrated a reduction in the severity of the disease, but he was unable to effect an eradication. TAUBENHAUS *et al.* (**11**) experimenting with flooding methods in Texas were also unable to eradicate the disease in the field, but found that mycelia survive only a few days in flooded soils in the labora-

tory. These findings may be explained by the results of KING (**6**), who found that the sclerotia stage of the organism was able to survive long periods of submersion.

Conditions of moisture and aeration in the field change considerably as the growing season progresses. With the drying out of the blackland (Houston and Wilson clay) soils of Texas, aeration is enhanced both by cultivation and the natural fissures that appear in the soil. Fissures serve as deep channels for the transport of gases and provide a means for exchange of oxygen and carbon dioxide at the deeper soil levels (**1**). The evidence reported here does not support the observation of GILBERT (**4**) that the fungus seems to grow best, and hence the disease is most severe, when the soil aeration is poorest.

Summary

An apparatus for the double culture of cotton plants and *Phymatotrichum omnivorum* in pure culture is described. Growth of the organism, and consequently infection, are directly correlated with the available oxygen supply. Application of these results to field observations concerning seedling infection and cultural practices is discussed.

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LEVULINS AND INULIN IN GUAYULE, *PARTHENIUM ARGENTATUM* A. GRAY[1]

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Recently McRARY and TRAUB (**16**) isolated a fructosan, or levulosan, tentatively identified as inulin, from guayule plants. Later HASSID *et al.* (**11**) definitely characterized this fructosan as inulin. Further work on the water soluble reserve carbohydrates of guayule has shown that in addition to inulin, levulins are also present, and in relatively greater amounts. The application of the principles developed in the present paper to physiological research problems by the authors and others (**39, 40, 3, 5, 6, 7**) has shown that the fractionation of the levulins into 89% ethanol soluble and insoluble portions may be used as a more dynamic tool in plant physiological research than the mere recording of total residues. The preliminary results concerning the free monosaccharides in guayule and the analytical methods for determining the water soluble carbohydrates in guayule are summarized elsewhere (**41, 39**).

According to McRARY and SLATTERY (**15**), continuous extraction, for six hours, of the finely ground guayule tissue when 80% ethanol by volume is placed in the boiling flask of the extraction apparatus (Landsiedl type), completely removes the "reducing sugars and sucrose" leaving the "fructosan" in the insoluble residue. Under the conditions indicated, it should be pointed out that the condensate dripping into the extraction cups is approximately 89% ethanol by volume (**9**) and is approximately at the boiling point of the solvent. In order to show that the interpretation of McRARY and SLATTERY (**15**) is untenable, previous work on the naturally occurring polysaccharides in some Compositae will be briefly reviewed, the water soluble carbohydrate reserves in guayule will be considered, and the fractions separated from guayule by McRARY and SLATTERY (**15**) will be critically examined.

Previous work

In 1870, POPP (**24**) reported a "soluble modification of inulin" in the tubers of *Helianthus tuberosus* which he named "inuloid." Since that date apparently the nature of the inulides (inuloid) or levulins in plants and their relation to inulin, and also the characteristics of inulin as it occurs in nature, were little understood until the subject was elucidated in 1932–1933 by SCHLUBACH and KNOOP (**30**).

Although TANRET (**35, 36**) in 1893 reported that he was able to separate from inulin associated matter (pseudo-inulin, mol. wt. 2610; inulenin, mol.

[1] Contribution from the Emergency Guayule Research Project, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture. The cooperation of the U. S. Forest Service is gratefully acknowledged.

wt. 1656; helianthenin, mol. wt. 1924; and synanthrin, mol. wt. 1314) having a lower specific rotation than inulin, neither DEAN (**8**) nor PRINGSHEIM and ARNOWSKY (**26**) could confirm these results. ÖHOLM (**23**) also showed on the basis of diffusion coefficients that the Kahlbaum preparation of inulin he used in his experiments apparently did not contain Tanret's reported synanthrin for the rates of diffusion were in harmony with STEFAN'S tables (**34**). On the assumption that Tanret's interpretation was valid, Öholm observed that the inulin sample apparently was not a pure one since it gave the same diffusion coefficient as TANRET'S (**35, 36**) postulated pseudo-inulin, mol. wt. 2612.

DEAN (**8**), in 1904, on the basis of work with *Dahlia variabilis, Helianthus tuberosus, etc.*, concluded that "Inulin is accompanied in these places [underground organs] by levulins, resembling inulin in composition, but differing from it in possessing a greater degree of solubility and a lower specific rotation." As a working hypothesis, Dean considered "the carbohydrates or carbohydrate mixture, which is readily precipitated in cold alcohol of 60% strength and has a specific rotation $[a]_D = -33°$ to $-40°$, inulin; and the undetermined mixture of lower rotatory power and greater solubility, the levulin mixture." WILLAMAN (**43**), in 1922, observed that on the basis of his experiments with *H. tuberosus*, Dean's theory with reference to the identity of inulin which postulates "the existence of various aggregates of molecules, more or less loosely combined; the greater the aggregate, the greater is its specific rotation, and the lower is its solubility in water . . . explains fairly well the behavior of inulin on successive crystallizations, either from water or from alcohol."

On the basis of results reported by SCHMIEDEBERG (**31**), MEYER (**18**), and GREEN (**10**), PRINGSHEIM (**31**) regards the "inulide which frequently accompanies inulin in nature . . . as a metabolic conversion product of inulin." He regards this fraction as "inulin-dextrins arising as a fermentative intermediate stage in plant metabolism," their occurrence depending on seasonal and other circumstances (**25, 27**). He cites the observation of POPP (**24**) that "a fructoside of this nature is polymerized to insoluble inulin on ageing, *e.g.*, in Helianthus nodules." GREEN (**10**) isolated an enzyme from sprouting girasole tubers, *H. tuberosus*, by extraction with glycerine, that split inulin into "a sugar and an intermediate body possessing properties which resemble those of inulin" but differs from inulin in such particulars as solubility in water and ethanol, and rate of dialysis.

In marked contrast to the conclusions of all previous workers, SCHLUBACH and KNOOP (**30**) report that the mixture of levulosans in the tubers of *H. tuberosus* apparently consists of three components only: inulin, a dilevan, and another di-D-fructose anhydride. According to these authorities there is no building up of inulin by polymerization of the lower polymer compounds, but each of the three is in turn built up directly from the common fundamental unit, fructose.

Recently MCDONALD (**17**) summarized the knowledge of well-defined

polyfructosans and difructose anhydrides. Consideration of similar or related compounds occurring in plants that are not as yet well defined is omitted but cannot be ignored by the physiologist. According to McDONALD (letter dated March 6, 1946), the "so-called levulins and inulides which occur along with inulin contain glucose as well as fructose, and hence, unless they are well-defined compounds, can only be referred to as polysaccharides." The term, "levulins," therefore, is used to refer in the usual sense to the mixture of non-reducing polysaccharides, in Compositae, containing mostly fructose and lesser proportions of glucose, soluble in cold water and associated with inulin which is not appreciably soluble in cold water.

LEVULINS AND INULIN IN GUAYULE

Using the cold and hot water extraction procedure of TRAUB *et al.* (**42**), the nature of the water soluble reserve carbohydrates in guayule was first tentatively identified as including cold water soluble levulins in addition to inulin. The fractions will be considered before the sucrose hypothesis of McRARY and SLATTERY (**15**).

INULIN IN GUAYULE TISSUE

Relatively pure inulin was isolated from finely ground guayule tissue according to the hot water extraction with subsequent freezing technique of SPOEHR (**33**). After the precipitation of inulin by freezing, the mass was thawed and inulin recovered by filtering on a Buechner funnel. The product, after three purifications, with an ash content of 0.49 ± .050%, on the basis of three determinations, had a specific rotation of $[a]_D 20 = -37.1°$ ± .17. This compares with $[a]_D 20 = -39.9°$ reported for *H. tuberosus* inulin by SCHLUBACH and KNOOP (**30**), $[a]_D\ 17 = -36.55°$ for inulin, source not indicated, by BERGMANN and KNEHE (**4**) and $[a]_D = -35°$ for guayule inulin, with an ash content of 1.2%, by HASSID *et al.* (**11**). The somewhat lower specific rotation, reported by HASSID *et al.* (**11**), apparently is due to the presence of some of the 89% ethanol insoluble levulins that were extracted with hot water along with the inulin, and were precipitated with the concentration of acetone used to bring down the inulin. The presence of these non-inulin substances in the inulin sample might also affect slightly the other properties reported for guayule inulin by the workers. SCHLUBACH and KNOOP (**30**) have discussed the difficulties encountered in the preparation of relatively pure inulin.

LEVULINS IN GUAYULE TISSUE

Levulins in the finely ground guayule tissue were separated quantitatively from inulin by extraction with water at room temperature, 23° C., (**39**), for at 20° C., water dissolves only 1 part of inulin in 10,000 (**19**). Analyses for total ketoses on the original filtrate from the Spoehr freezing method (**33**), and on the cold water extracts, after clarification in the usual

manner (**39**), and subsequent hydrolysis with 1% HCl, gave reducing values for ketoses of over 95% of the total non-reducing substances present. The cold water soluble polysaccharides in guayule tissue may consist mainly of difructose anhydrides or polyfructosans. If sucrose be present, it would constitute a minor fraction of the whole. Work reported elsewhere (**39, 40**) shows that the values for levulins in guayule tissue are greater than those for inulin. The reserve polysaccharides in guayule from the standpoint of solubility in cold and hot water therefore are similar to those found in the tubers of the girasole, *H. tuberosus*, also a Composite, as previously reported by WILLAMAN (**43**) and TRAUB *et al.* (**42**).

The final identification of the components of the levulins fraction in guayule tissue presents a major chemical research problem, and remains to be carried out. It was possible, however, to conduct preliminary experiments for the purpose of determining whether the components of the levulins fraction form an homologous series of polymers intermediate between inulin and the monosaccharides present in the tissues as postulated by PRINGSHEIM *et al.* (**25**) and others; or, whether the conclusions of SCHLUBACH and KNOOP (**30**), based on *H. tuberosus*, apply to guayule tissue. The latter authors report the presence of only two difructose compounds, dilevan and another difructose anhydride in addition to inulin in the tubers of *H. tuberosus*. The subject may be explored in a preliminary way by determining diffusion coefficients and molecular weights of the levulins extracted with various concentrations of ethanol.

ÖHOLM (**22, 23**) has pointed out that the approximate molecular weights of compounds, including non-electrolytes, may be calculated from diffusion coefficients, and that the method is applicable in cases where the freezing point depression method does not apply, in the case of colloids for instance, where the presence of slight impurities of electrolytes would give erroneous results. MEYER (**19**) has emphasized the adequacy of such approximate values in cases where relative ones will suffice.

The approximate molecular weights of the levulins fractions extracted with various concentrations of ethanol were determined by the diffusion method, using the apparatus and procedure described by ÖHOLM (**21**). Guayule tissue, prepared as described by TRAUB (**37**), was first extracted continuously for six hours in a reflux (Landsiedl type) apparatus by placing 100% ethanol in the boiling flask, in order to remove the free sugars. The extract was analyzed for free-reducing and non-reducing substances (table I). Since the tissue contained a slight amount of moisture, the condensate after extraction began was 97% ethanol by volume, and if present, any sucrose (approximately 0.59% soluble in 100% ethanol at room temperature), as well as levulins, was extracted. The residue was then extracted successively with 92%, 89%, and 50% ethanol by volume. For this purpose the required concentrations of ethanol, from EVANS' table (**9**) were placed in the boiling flasks. The final residue, after extraction with the ethanol-water mixtures, was then successively extracted with cold and hot water (nos. 5

and 6 in table I). All extractions were made in the presence of calcium carbonate to prevent hydrolysis of polysaccharides. In the case of extracts nos. 2, 3, and 4, the ethanol was evaporated, and the residue taken up in water (table I). Proteins, pectins, tannins, *etc.*, were precipitated from all of the extracts with neutral lead acetate, and the solutions were deleaded and clarified. Aliquots of the clarified extracts, 10-ml., were then introduced into the diffusion apparatus for the determination of the actual amounts diffused at constant temperature, 22° C., in the four aqueous layers of uniform depth. The levulins in the original extracts (nos. 2, 3, 4, and 5, table I), and in the aqueous layers from the diffusion apparatus at the end of the diffusion periods, were determined after hydrolysis as

TABLE I

APPROXIMATE DIFFUSION COEFFICIENTS (D_{22}) AND MOLECULAR WEIGHTS FOR LEVULINS FRACTIONS EXTRACTED FROM GUAYULE TISSUES CONTAINING 13.1% POLYSACCHARIDES: 12.6% LEVULINS AND 0.5% INULIN

Fraction number	Ethanol extract*		Water extract		Dry weight of tissue		D_{22}	Mol. weight*
		Fraction no. residue	Temperature	Fraction no. residue	Levulins†	Inulin†		
	%		C.		%	%		
1	97	.....	.....	.....	2.9‡	0.0	‡	‡
2	92	1	.....	.....	3.5	0.0	0.328	521
3	89	2	.....	.....	0.7	0.0	0.334	503
4	50	3	.....	.....	5.5	0.0	0.249	905
5	.....	.....	20	4	Trace	0.0	.....	.....
6	.....	.....	100	5	0.0	0.5	§	§

* The results are reproducible within 8% of the values indicated. The difference between nos. 2 and 3 is therefore not significant, but the difference of either no. 2 or no. 3 as contrasted with no. 4 is significant.

† Expressed as fructose. Similar values for a tissue containing 4.74% levulins, and 3.89% inulin, are: (no. 1) 1.16; (no. 2) 1.20; (no. 3) 0.27; (no. 4) 3.89; (no. 5) trace; and (no. 6) 3.89.

‡ The total 97% ethanol extract contained 3.2% carbohydrates, including 0.3% free sugars, which was subtracted. Due to the presence of free sugars it was not practicable to determine the diffusion coefficient and molecular weight for non-reducing substances in this fraction.

§ Compare mol. wt. of 5000 for inulin reported by HAWORTH *et al.* (12).

total reducing substances expressed as fructose by the SOMOGYI method (**32**). The relative distribution of the levulins in the four aqueous layers was calculated from the actual amounts present, and the diffusion coefficients were obtained by reference to diffusion tables of SCHEFFER (**29**) and KAWALKI (**14**) as compiled by ARNDT (**2**). Molecular weights were calculated from the following equation: $\sqrt{M} = \frac{7 \times 1.07}{D_{22}}$. Results from a typical experiment are given (table I).

HAWORTH, HIRST AND PERCIVAL (**12**) determined the end groups of inulin as 1,3,4,6-tetramethyl-fructopyranose, and concluded that the inulin mole-

cule consists of a chain having a minimum average length of 30 D-fructose units, and a molecular weight of about 5000. The levulins fractions obtained from guayule tissue by extraction with various concentrations of ethanol have lower molecular weights than that reported for inulin (table I). The results also indicate that the polysaccharide components may be approximately separated into two major parts by extraction of guayule tissue with 89% ethanol, under the conditions indicated, and that the 89% ethanol soluble levulins have lower molecular weights than those which are insoluble in the same solvent. Apparently, these results do not corroborate the hypothesis of PRINGSHEIM (**25**), and others, for an homologous series of inulides or levulins between inulin and the monosaccharides, nor do they indicate that difructose anhydrides are components of the guayule levulins fraction. Only further research can throw additional light on this subject.

HYDROLYSIS OF LEVULINS

After evaporating the ethanol, MCRARY and SLATTERY (**15**) subjected the residues from 89% ethanol soluble extracts of guayule tissue to hydrolysis with bottom invertase, and after subtracting the values for free-reducing substances from those of the total 89% ethanol soluble carbohydrates, reported the difference as "invert sugar from sucrose." In the experiment, however, it was found that hydrolysis of the 89% ethanol extract residue from guayule tissue with top invertase, or with 1% HCl, gave reducing values very much greater than were obtained with bottom invertase (table II). Hydrolysis with top invertase gave values ranging from 30% to 65% higher than those obtained with invertase of bottom fermentation. The corresponding values for 1% HCl are slightly higher than those indicated for top invertase. It may be of interest to note that ADAMS, RICHTMYER and HUDSON (**1**) report that the pH conditions for the maximum hydrolysis of sucrose, raffinose and inulin differ, and conclude that a specific inulase rather than a β-D-fructofuranosidase is responsible for inulin hydrolysis

TABLE II

COMPARISON OF EFFECTIVENESS OF BOTTOM AND TOP INVERTASES AND 1% HCl IN HYDROLYZING NON-REDUCING SUBSTANCES FROM RESIDUE OF 89% ETHANOL EXTRACT OF GUAYULE TISSUE

SAMPLE NUMBER	NON-REDUCING SUBSTANCES*			INCREASE, TOP OVER BOTTOM INVERTASE
	HYDROLYSIS			
	BOTTOM INVERTASE	TOP INVERTASE	1% HCl	
	%	%	%	%
1	4.91	7.84	8.23	59.7
2	4.29	7.09	7.86	65.3
3	4.67	7.59	8.04	62.5
4	2.03	2.64	2.79	30.0

* Results expressed on basis of total dry weight of tissue.

when yeast invertases are used. On the basis of the data presented, it is safe to conclude that the major part of the 89% ethanol soluble polysaccharides in guayule is not sucrose, for the use of bottom and top invertases, under the usual procedure for the hydrolysis of sucrose resulted in only partial hydrolysis of the total present in the tissues.

It should be noted that when the solutions of the 89% ethanol soluble residues were hydrolyzed with 1% HCl, the values for ketoses by ROE's method (**28**) as modified by MCRARY and SLATTERY (**15**) ranged from about 94% to 96% of the total 89% ethanol soluble non-reducing substances (table III). Similar values for fructose by NYNS' method (**20**) as modified by

TABLE III

PROPORTION OF KETOSES TO TOTAL NON-REDUCING SUBSTANCES. (RESIDUE OF 89% ETHANOL EXTRACT HYDROLYZED WITH 1% HCl)

SAMPLE NUMBER	NON-REDUCING SUBSTANCES*	KETOSES*†	PROPORTION OF KETOSES TO NON-REDUCING SUBSTANCES‡
	%	%	%
1	8.23	7.76	94.3
2	7.86	7.51	95.5
3	8.04	7.79	96.9
4	2.78	2.64	95.0

* Expressed on basis of total dry weight.

† Total ketoses by ROE's method (28) as modified by MCRARY and SLATTERY (15), less fructose of free sugars by NYNS' method (20) as modified by JACKSON and MATHEWS (13). Ketose values include any ketoses, other than fructose, if such constitute a portion of the total free sugars TRAUB, SLATTERY and WALTER (41).

‡ Expressed on basis of total non-reducing substances.

JACKSON and MATHEWS (**13**) agreed with those for total ketoses. Again sucrose as the major polysaccharide originally present in the tissues is ruled out. The relatively high ketose values point to levulins as the water soluble polysaccharides associated with inulin in guayule tissues. The 89% ethanol extract, however, apparently represents only part of the total levulins (table I). MCRARY and SLATTERY (**15**) are therefore not justified in assuming that extraction with 89% ethanol under the conditions stated is a valid criterion for the quantitative separation of "fructosan" from some other non-reducing substance in guayule tissues.

FRACTIONATION OF LEVULINS FOR PHYSIOLOGICAL STUDIES

It has already been noted in the discussion of the results reported in table I that extraction of guayule tissues with 89% ethanol effectively separates the levulins into two major portions. The use of this procedure may therefore serve as a convenient sieve to distinguish changes in the two fractions with time. On successive dates such changes may be determined and correlated with such environmental factors as available mineral nutrients, light intensity and duration, CO_2 content of the air, or with low temperature, soil moisture stress (drought), *etc.* Experiments have shown that

the absolute and relative proportions of the two levulins fractions in guayule plants are markedly affected by low temperature, high soil moisture stress, and other environmental factors (**3, 5, 6, 7, 38, 39, 40**). The making of such measurements has proved to be a more dynamic, and therefore a more valuable tool in physiological research than the analysis of total cold water soluble polysaccharides alone.

Role of carbohydrate fractions

On the basis of the available facts, inulin, insoluble in water at ordinary temperatures, is the least available storage form of carbohydrates in the guayule plant, while levulins, soluble in water at ordinary temperatures, are the more readily available reserve carbohydrates in solution in the cell sap. Fructose, or levulose, and other free monosaccharides (**7, 39, 41**), apparently are the chief carbohydrates of translocation since they are always present in relatively small amounts in the stems and roots of the guayule plant, but predominate in the leaves and inflorescences. However, there is the possibility that levulins may also be translocated.

Summary

1. The presence of levulins in guayule tissues in addition to inulin previously reported, and in relatively greater amounts, has been established. Thus the McRary-Slattery assumption that sucrose is a major reserve carbohydrate associated with inulin in guayule tissues has been shown to be untenable. If sucrose should be present at all, it would constitute only a minor fraction of the total carbohydrate reserves.

2. The levulins may be fractionated into two major portions by extraction of guayule tissues with 89% ethanol: (a) the 89% ethanol soluble fraction, and (b) the 89% ethanol insoluble fraction. On the basis of this fact, a method has been devised for use in seasonal physiological studies to determine the absolute and relative proportions of the two fractions in response to environmental factors. The method is a more dynamic tool for such studies than the mere recording of total residues.

3. On the basis of diffusion coefficients, it has been shown, on the one hand, that Pringsheim's hypothesis for the presence of an homologous series of polymers between inulin and the monosaccharides does not hold for guayule; and on the other hand, that apparently difructose anhydrides are not present in guayule plants although reported in girasole tubers, *Helianthus tuberosus,* also a Composite, by Schlubach and Knoop.

4. The levulins are the chief reserve carbohydrates in the guayule plant, and inulin is secondary in importance from this standpoint.

5. Although, under the experimental conditions, top invertase hydrolyzed from 30% to 65% more of the levulins extracted from guayule tissues than were hydrolyzed by invertase of bottom fermentation, hydrolysis with 1% HCl was required to obtain complete hydrolysis of the levulins.

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AN AUTOMATIC APPARATUS TO COVER AND UNCOVER PLANTS IN PHOTOPERIOD EXPERIMENTS

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(WITH ONE FIGURE)

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In photoperiod experiments short-day plants are usually either covered with cloth shades hung over some sort of framework or else placed in light-

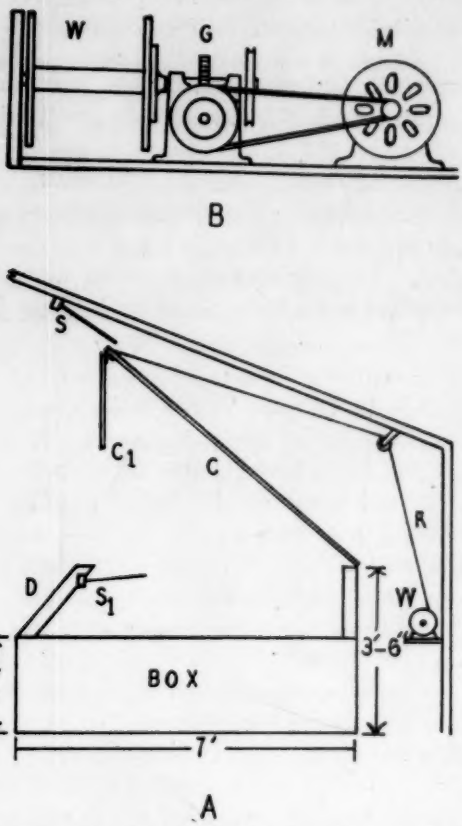

FIG. 1. (A) Diagrammatic end view showing arrangement of box and winch in relation to side wall and roof of greenhouse. (B) Arrangement of motor, speed reducing gear, and winch. The drum of the winch is a piece of hard wood with the outer end turned down to form a shaft. This is supported in a hole in a board attached to the base and lubricated with oil and graphite. The ends of the drum are discs of plywood, and the inner end is bolted to a plate on the reducing gear.

proof boxes. Whatever the details, these methods require someone to be present at a specific time each morning and evening to attend to the plants. Difficulty in finding anyone to do this led to the development of a mechanical

apparatus to cover and uncover groups of plants at any predetermined time, without requiring the presence of an attendant.

The apparatus consists of a box-like enclosure seven feet square, with front and sides two feet high and a back three and one-half feet high (fig. 1). The cover is a light wooden frame (C) hinged to the back and covered with light-proof cloth which hangs down on the sides and front overlapping the wooden sides of the box and darkening the interior. The part of the cover overhanging the front (C_1) is mounted on a framework hinged to the top frame allowing it to swing back against the top when the cover is raised, thereby reducing shading of the interior of the box. The cloth forming the sides is attached to the front of the cover and to the back of the box so that the cloth is firmly held in place when the cover is down. Overhead clearance of about 10 feet is desirable to permit raising the cover almost to the vertical position during the day to prevent shading the plants. When the cover is lowered the hinged front slides down the sloping posts (D) at the front corners of the box. This prevents it from being caught on the posts or walls in such a way as to prevent complete closure. If the box faces approximately south, the cover can be pulled back toward the north where it intercepts the minimum of light.

Boxes of various sizes can be constructed. Larger boxes should be rectangular with the cover hinged on the long edge as too much head room is required for a cover larger than six or seven feet wide. Under some conditions a ventilator fan would be necessary to prevent the temperature inside the closed box from becoming much higher than outside. This can be controlled by a switch operating only when the cover is down.

The position of the cover is controlled by an electric time switch through which current is supplied to a reversible electric motor (M). The motor drives a 48 to 1 reduction gear (G) to which is attached a small wooden winch (W). The winch raises and lowers the cover by winding and unwinding a rope (R) extending from the cover over a pulley attached to the greenhouse roof and down to the winch. The time switch has two separate circuits, controlled independently, one to raise the cover in the morning, the other to lower it at night. One of the circuits operates a double-pole double-throw relay used as a reversing switch, so the motor can be operated in one direction to lower the cover and in the opposite direction to raise it. Limit switches (S and S_1) are so placed that when the cover reaches the fully open and fully closed positions it operates the switches, breaking the circuit and stopping the motor. Limit switch S_1 is placed on the inside of post D and can be operated by a wire projecting from the cover.

A motor of one-sixth horsepower is used in this apparatus, but a one-eighth horsepower motor could lift a cover of the same size. The reduction gear is of the small worm gear type commonly listed in catalogs of scientific apparatus. The limit switches are small toggle switches with extensions made of copper tubing fastened to the metal toggles. They are held in the closed position by gravity and springs except when the cover is pressed

against the toggle extensions. The time clock controls the current from the line to one side of the motor through two separate circuits. One circuit (A) is used to supply current in the morning to lift the cover, the other circuit (B) supplies current to replace the cover in the evening. The sequence of events is as follows: in the morning the time clock closes circuit A, the motor starts, and the cover is lifted until it strikes the limit switch S, stopping the motor. In a few minutes the time clock turns off the current in circuit A. Removal of the cover has meanwhile allowed switch S_1 to close, making circuit B available for use in the evening. At the desired time in the evening the time switch turns current on in circuit B, actuating the reversing switch and running the motor and winch in reverse to lower the cover. As the cover is lowered switch S closes so that circuit A is ready for operation the next morning. When the cover is in the closed position it opens switch S_1 stopping the motor.

Many of the details of this apparatus are susceptible to variation, and ingenious readers will doubtless think of various improvements. The apparatus as described has been in operation over a year with only minor mishaps and has not failed once in the past six months. It provides a practical and convenient method of growing plants with short photoperiods for research and class use.

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NOTES

John Wesley Shive.—Dr. Shive has retired from active professional work this year. He relinquished his duties both at Rutgers University where he has been Head of the Botany and Plant Pathology Department since 1924 and at the New Jersey Experiment Station in Brunswick where he has been Head of the Department of Plant Physiology.

During the thirty-one years Dr. Shive has been at Rutgers University and at the New Jersey Experimental Station, he has been an outstanding teacher of plant physiology. He is an inspiring and conscientious instructor as evidenced by the great number of his students who have continued in teaching and research in plant physiology and related fields.

Dr. Shive is an analyst by nature and is extremely skillful in developing a productive research. He is a scholarly thinker and a sound logician, readily detecting fallacies in unwarranted conclusions. He himself has unusual ability and enthusiasm for research work, and he has been quick to recognize and encourage the efforts and contributions of his students. He has directly supervised the research projects of over fifty graduate students. In addition to the help he has given students in publishing their scientific papers, he also is the joint author of fifty-two papers and the sole author of some forty independent scientific works.

John Shive was born in Halifax, Pennsylvania, in 1877. He received his Ph.B. in 1906 and his A.M. in 1908, both from Dickinson College. From 1913 to 1915 he was a fellow in plant physiology at Johns Hopkins, and in 1915 he received his Ph.D. degree from that institution.

During his professional career, Dr. Shive has achieved international distinction and enjoys a position of highest esteem among his American colleagues. He collaborated with a special Committee on the Division of Biology and Agriculture of the National Research Council under the chairmanship of Dr. B. E. Livingston in formulating and testing standard series of nutrient solutions for plant culture. The comprehensive report of the Committee was published in Baltimore in 1919 as "A Plan for Cooperative Research on the Salt Requirements of Representative Agricultural Plants." He is Associate Editor of *Soil Science*, journal of the Torrey Botanical Club, and a member of the Editorial Board of *Plant Physiology;* he is a charter member and past president of the American Society of Plant Physiologists. In 1938 the latter organization awarded him the honorary Stephen Hales Prize "in recognition of a lifetime of distinguished service to plant physiology." He has been cited and elected to membership in the Royal Agricultural Society of Sweden and in 1938 he was starred in "American Men of Science." He is a member of Torrey Botanical Club and the Botanical Society of America; and he has been elected to honorary membership by the Rutgers Chapters of Phi Beta Kappa and Sigma Xi, serving as Chapter president of each, and of Alpha Zeta.

Dr. Shive's interests and abilities are varied. Not only is he an expert scientist; he is also a photographer of professional grade, a skillful craftsman of great originality in experimental work, an outdoor enthusiast, and a participant in numerous sports. The American Society of Plant Physiologists and its journal, *Plant Physiology*, are pleased to acknowledge his distinguished contributions as a scientist, as an officer of many years of able service, and as a loyal friend of the profession.

Joseph H. Gourley.—Dr. Gourley, Head of the Department of Horticulture at the Ohio Agricultural Station since 1921 and Chairman of the Department of Horticulture and Forestry at the Ohio State University since 1929, died at the age of 63 on October 27, 1946, at Wooster, Ohio, following an attack of coronary thrombosis. Until a week before his death he was carrying heavy administrative responsibilities, in addition to the direction of a large class of senior and graduate students and the instruction of 165 freshman majors in horticulture.

The entire field of plant science suffers a great loss in the death of Dr. Gourley, for in addition to outstanding contributions to research in his own field, such as his orchard soil management studies, he possessed a broad knowledge and a stimulating interest in plant physiology and plant anatomy, the subject in which he obtained his doctor's degree at the University of Chicago. He believed deeply in the establishment and maintenance of the closest possible relations between the various fields of plant science, and he felt that horticulture could be adequately served only so long as its investigators and teachers possessed thorough basic training in botany and plant physiology. This breadth of viewpoint made him keenly aware of new developments throughout the field so that men doing research under him were always encouraged to advance along new and exploratory lines. Dr. Gourley, who was well known throughout the country, will be greatly missed by all who knew him, not only as a scientist and teacher, but also as a person of warmth and charm with an unlimited capacity for friendship.

Bayard F. Floyd.—On November 8, 1945, Mr. Floyd, Vice-President of the Wilson and Toomer Fertilizer Company of Davenport, Florida, died of a cerebral hemorrhage. He was stricken while attending an all-day meeting of the Florida Agricultural Research Institute in Winter Haven, Florida. He is survived by his widow, Mrs. Dixie W. Floyd.

Walter S. Clark.—Probably the youngest member of the American Society of Plant Physiologists, Mr. Walter S. Clark died on October 31, 1946. During the past three and one-half years he has been assisting in the Department of Botany at Duke University. His exceptional interest and outstanding ability in science gained him an early admittance to the University be-

fore his high school graduation. He has published one paper and was co-author of another which is now in press. His high scholarship was recognized in his junior year by his election to Phi Beta Kappa. MR. CLARK is survived by his mother, MRS. FRANCES CLARK BRITTON, of Miami, Florida.

Life Membership.—The Executive Secretary-Treasurer has announced the purchase of a Life Membership by DR. WENDELL R. MULLISON. DR. MULLISON, recently returned from Curacao, is now Plant Physiologist with the Dow Chemical Company at Midland, Michigan. The Society is very happy to welcome DR. MULLISON to the growing roster of Life Members.

The Society offers a Life Membership on the payment of One Hundred Dollars in a lump sum. Life members pay no further dues and they receive the journal for life without any additional charge. Members may become Patrons of the Society upon payment of Two Hundred Dollars (or more at their option) and also receive the journal for life without further cost. A Life Membership represents a sound financial investment as well as the satisfaction of professional distinction.

Crop Production and Environment.—R. O. WHYTE. Faber and Faber, Ltd., 24 Russell Square, London, W. C. 1, England. Ryerson Press, Agent, 299 Queen Street, West Toronto (2B), Canada. 372 pages. $5.00.

The author's long association with the Imperial Bureau of Pastures and Forage Crops at Aberystwyth, Wales, makes him well qualified to undertake a correlation of research in pure and applied science as related to crop production. The subject is approached from the standpoint of development. The terms *growth* and *development* are differentiated on the basis of definitions given at the outset by the author. The first portion of the book critically reviews recent important researches on environmental factors in relation to growth and development, especially as they influence growth substances and nutrients as autogenous factors in ontogeny. The final chapters are devoted primarily to a consideration of the application of the foregoing physiological principles to the production of particular crops. Special attention is given to physiological aspects of the geographical distribution of crop plants, plant introduction, and breeding. The book concludes with a survey of the developmental behavior of crops and methods of manipulation of their growth and development.

The comprehensiveness of the world literature reviewed is correlated in an exceptionally effective manner and makes the book particularly valuable. Despite the handicaps of war, the author appears to have had access to recent publications of many nations and thus brings the discussions up to the present date. Readers will find the thorough appraisal of recent Russian researches particularly illuminating. The problems of production of specific crops are of as much interest to plant physiologists as to agronomists. The historical review of physiological research on growth and development

beginning with the work of Klebs vividly reveals the epoch-making strides made in the brief period of less than three decades. These studies are still gaining in momentum and pace, suggesting that even greater success in understanding and control of life processes in plants may be expected within the next few years.

The subject matter of the book is arranged as a very logical progression of ideas. Figures and tables have been judiciously selected and contribute considerably to clarification of the discussions. Particular attention has been given to citations and indexes which facilitate use of the book for rapid reference. Citations are grouped by chapters but are supplemented by separate volume indexes of authors, genera, and subjects. A very helpful glossary of scientific terms is also included.

Ernährung und Stoffwechsel der Pflanzen. — A. FREY-WISSLING. Büchergilde Gutenberg, Zurich, Switzerland. G. E. Stechert & Company, 31 East 10th Street, New York 3, New York. 295 pages. $2.50.

Plant scientists will welcome this authoritative monograph of plant nutrition and metabolism. The content is grouped under the following major headings: Resorption, Assimilation, Translocation and Accumulation, Growth, Dissimilation, and Elimination. Though the text presupposes a knowledge of physical and chemical fundamentals, it is not extremely technical. The author brings up-to-date the established concepts concerning the topics discussed and includes reference to much recent European research which has been relatively inaccessible on account of the War. In view of the wartime difficulties under which the book was written, it is excellently documented.

With the exception of the comparatively brief discussion of recent work on photosynthesis, all topics are rather comprehensively presented. The author's special competence concerning the physiology of elimination by plants makes this section of the book a very helpful and critical summary. The section on translocation emphasizes important recent researches on the conduction of water and solutes through cell walls. The work of Wieland and Warburg on respiration is reconciled in a clear and concise manner. The book has an attractive and very readable format. It has separate author and subject-matter indexes, the latter being quite comprehensive and thoroughly cross-indexed. Numerous figures and tables contribute considerably to the clarity of the contents.

Contribution to the Physics of Cellulose Fibres.—P. H. HERMANS. Elsevier Publishing Company, New York, New York. 225 pages. $4.00.

This book on sorption, density, refractive power, and orientation of cellulose fibers constitutes communication no. 21 of the Institute for Cellulose Research of the Algemeene Kunstzijde Unie (A.K.U.) of Utrecht, Holland, and is one of the monograph series, Progress of Research in Holland during the War. Though endeavoring to assemble the results of earlier

related research, much of this publication is an original contribution on the physical properties of cellulose.

Though the subjects discussed are of interest primarily to those engaged in cellulose and fiber research, the interpretations of density measurements and optical properties make the subject matter of value to plant scientists. A special section on experimental methods is included. The differences between native and regenerated fibers in sorptive properties and swelling capacity are given special emphasis. The various sections on the theoretical background of cellulose physics are each concluded by a very concise and useful summary. Though the book lacks a general index, the table of contents is sufficiently detailed to permit use for reference purposes.

Goethe's Botany; The Metamorphosis of Plants (1790) and Tobler's Ode to Nature (1782).—Edited by FRANS VERDOORN. Chronica Botanica Company, Waltham, Massachusetts. 63 pages. $2.00.

Botanists will welcome the translation into English of Goethe's Metamorphosis of Plants and the accompanying critique of his concepts by a very competent reviewer. The origin of Goethe's interest in plants and his general viewpoint of metamorphosis are of special interest. This concise review is authentically documented and written in a very lucid style.

pH and Plants.—JAMES SMALL. Baillière, Tindall and Cox, 7 and 8 Henrietta Street, Covent Garden, London, England; G. E. Stechert & Co., 31 East 10th Street, New York 3, New York. 216 pages. $4.00.

This compact book reviews recent important methods and data on pH in relation to plant physiology. Methods of approach, applications, and types of results expounded provide a helpful résumé of the current status of hydrion studies. A wide variety of data are included on interrelationships between pH and such topics as metabolism, enzymatic reactions, plant structures, pathology, and ecology. The book is well illustrated, and a selected bibliography of over 300 important papers is included.